NOVEL ENOYL REDUCTASES AND METHODS OF USE THEREOF

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FIELD OF THE INVENTION

The present invention relates to novel enzymes that act as enoyl reductases. Two distinct families of enoyl reductases have been identified in bacteria, each of which have a consensus amino acid sequence. The enoyl reductases can be used as targets for designing both new prophylactics and treatments for bacterial infections. Nucleic acid and amino acid sequences of the novel enoyl reductases are also provided.

BACKGROUND OF THE INVENTION

Essentially all living organisms synthesize saturated fatty acids by the same biochemical mechanism. However, whereas vertebrates and yeast synthesize saturated fatty acids using either one or two multifunctional enzymes (*i.e.*, type I fatty acid synthases, FASs), with the *acyl carrier protein* (ACP) being an integral part of the complex, most bacteria and plants synthesize saturated fatty acids through the use of a set of distinct enzymes that are each encoded by an individual gene (*i.e.*, type II FASs). In the type II FAS system, ACP is also a distinct protein.

The initial step in the biosynthetic cycle of saturated fatty acids is performed by the enzyme FabH [Tsay *et al.*, *J. Biol. Chem.* **267**:6807-68014 (1992), and U.S. Patent No: 5,759,832, Issued June 2, 1998, both of which are hereby incorporated by reference in their entireties] which catalyzes the condensation of malonyl-ACP with acetyl-COA. Malonyl-ACP is condensed with the growing-chain acyl-ACP in

subsequent rounds by FabB synthase I or by FabF, synthase II. The next step is a ketoester reduction that is catalyzed by an NADPH-dependent β-ketoacyl-ACP reductase (FabG). A β-hydroxyacyl-ACP dehydrase (FabA, dehydrase I or FabZ, dehydrase II) catalyzes the subsequent dehydration forming *trans*-2-enoyl-ACP. FabI, an NADH-dependent enoyl-ACP reductase, then catalyzes the conversion of *trans*-2-enoyl-ACP to acyl-ACP to complete the elongation cycle. The addition of two carbon atoms per elongation cycle continues until palmitoyl-ACP is synthesized. Palmitoyl-ACP is one end-product of the pathway and acts as a feedback inhibitor for both FabH and FabI [Heath, *et al*, *J.Biol.Chem.* **271**:1833-1836 (1996)].

Since an enoyl-ACP reductase catalyzes the final step in the biosynthetic pathway of saturated fatty acids, it is not surprising that it is also a key regulatory target for the pathway [Heath, and Rock, *J.Biol.Chem.* **271**:1833-1836 (1996); Heath and Rock, *J.Biol.Chem.* **271**:10996-11000 [(1996)]. Thus, pharmaceutical companies have placed considerable effort toward developing drugs that inhibit enoyl-ACP reductases and/or the reactions they catalyze. For example, the enoyl-ACP reductase of *Mycobacterium tuberculosis* (InhA) is a target for the drug isonaizid [Banerjee *et al.*, *Science*, **263**:227 (1994)] whereas, both diazaborines [Baldock *et al.*, *Biochem. Pharmacol.*, **55**:1541 (1998)] and triclosan [McMurray *et al.*, *Nature* (London), **394**:531 (1998) and Heath *et al.*, *J. Biol. Chem.*, **273**:30316 (1998)] inhibit the *Escherichia coli* enoyl-ACP reductase, FabI. All three drugs act through the formation of a high-affinity enzyme-NAD^+-drug ternary complex [Heath *et al.*, *J. Biol. Chem.*, **274**:11110-11114 (1999) and Rozwarski *et al.*, *Science*, **279**:98 (1998); Baldock *et al.*, *Science*, **274**:2107 (1996); Levy *et al.*, *Nature (London)* **398**:383 (1999); Stewart et al, *J. Mol. Biol.*, **290**:859 (1999); and Ward *et al.*, *Biochemistry*, **38**:12514 (1999)]. Consistently, missense mutations resulting in single amino acid substitutions in the active sites of the enoyl-ACP reductases prevent the formation of the ternary complexes and confer a resistant phenotype to bacteria expressing the mutant proteins [Banerjee *et al.*, *Science*, **263**:227 (1994); McMurray *et al.*, *Nature (London)*, **394**:531 (1998); Heath *et al.*, *J. Biol. Chem.*, **273**:30316 (1998); Heath *et al.*, *J. Biol. Chem.*, **274**:11110-11114 (1999); and Bergler *et al.*, *J. Gen. Microbiol.*,

138:2093 (1992) and Rouse *et al.*, *Antimicrobiol. Agents. Chem.*, **39**:2472 (1995)].

Unfortunately, the toxicity of boron severely limits the pharmaceutical application of diazaborines [Baldock et al, *Biochem. Pharmacol.*, **55**:1541 (1998)]. Triclosan, on the other hand, is widely employed as an antibacterial in consumer products for external use. Triclosan is a diphenyl ether (bis-phenyl) derivative, known as either 2,4,4'-Trichloro-2'-hydroxydiphenyl ether or 5-Chloro-2-(2,4-dichlorophenoxy) phenol, and is used as an antibacterial in antimicrobial creams, antiperspirants, body washes, cosmetics, deodorants, deodorant soaps, detergents, dish washing liquids, hand soaps, lotions, and toothpaste, as well as in plastics, polymers and textiles [*see*, Bhargava and Leonard, *Am. J. Infect. Control*, **24**:209 (1996)]. However, the hydrophobic nature and chlorine content of triclosan makes it undesirable for internal use.

Bacterial infections remain among the most common and deadly causes of human disease. For example, Streptococci are known to cause otitis media, conjunctivitis, pneumonia, bacteremia, meningitis, sinusitis, pleural empyema and endocarditis. In addition, virulent strains of *E. coli* can cause severe diarrhea, a condition which worldwide kills a million more people (3 million) every year than malaria [D. Leff, *BIOWORLD TODAY*, **9**:1,3 (1998)]. Indeed, infectious diseases are the third leading cause of death in the United States and the leading cause of death worldwide [Binder *et al.*, *Science* **284**:1311-1313 (1999)].

Although, there was initial optimism in the middle of the 20th century that diseases caused by bacteria would be quickly eradicated, it has become evident that the so-called "miracle drugs" are not sufficient to accomplish this task. Indeed, antibiotic resistant pathogenic strains of bacteria have become common-place, and bacterial resistance to the new variations of these drugs appears to be outpacing the ability of scientists to develop effective chemical analogs of the existing drugs [*See*, Stuart B. Levy, The Challenge of Antibiotic Resistance, in *Scientific American,* 46-53 (March, 1998)]. Therefore, new approaches to drug development are necessary to combat the

ever-increasing number of antibiotic-resistant pathogens.

Classical penicillin-type antibiotics effect a single class of proteins known as autolysins. Therefore, the development of new drugs which effect an alternative bacterial target protein would be desirable. Such a target protein ideally would be indispensable for bacterial survival. Thus the identification of a new bacterial enzyme that is required for fatty acid synthesis would be a prime candidate for such drug development.

Therefore, there is a need to identify new proteins that have enzymatic activities that are crucial for bacterial growth. There is also a need to provide immunogenic compositions containing such enzymes or fragments thereof. In addition, there is a need to develop methods for identifying drugs that interfere with such enzymes. Finally, there is a need to employ such procedures to develop new anti-bacterial drugs.

The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

SUMMARY OF THE INVENTION

The present invention provides two families of enzymes that can act as enoyl reductases. One such family shares a common amino acid consensus sequence, SEQ ID NO:45 and binds a flavin cofactor. This family of enoyl reductases is exemplified by the *Streptococcus pneumoniae,* FabK having an amino acid sequence of SEQ ID NO:2 and is naturally encoded by SEQ ID NO:1, as disclosed herein. The other family of enoyl reductases shares a common amino acid consensus sequence, SEQ ID NO:57 and like the previously disclosed FabI does not contain a flavin cofactor. This second family of enoyl reductases is exemplified by the *Campylobacter jejuni* FabL having an amino acid sequence of SEQ ID NO:52 and is naturally encoded by SEQ ID NO:51, as disclosed herein.

As disclosed herein and exemplified below, bacteria can express either or both of two unique enoyl reductases, FabK and/or FabL each of which catalyze the identical reaction as the well-characterized Gram-negative bacterial enoyl-ACP reductase, FabI. Since FabI has been a useful target for the design of antibacterials, the identification of FabK and FabL provides another important target. Indeed, the disclosure of FabK and FabL and their related analogs should have a major impact on the development of new prophylactics and treatments for bacterial infections, including those pharmaceuticals that can be used to combat antibiotic resistant *Streptococcus* and *Enterococcus strains*.

Thus the present invention provides an isolated nucleic acid that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:45. The present invention further provides an isolated nucleic acid that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:57. Preferably the polypeptide acts enzymatically as an enoyl reductase. In the case of FabK enoyl reductases, such nucleic acids preferably encode a polypeptide that also binds a flavin prosthetic group. Although the enoyl reductase can be obtained form any source, particularly from fungus, bacteria or plants, in a preferred embodiment the enoyl reductase is not a yeast enzyme. More preferably, the polypeptide is a bacterial enzyme or an active fragment of the bacterial enzyme. The polypeptides encoded by the nucleic acids are also part of the present invention.

In one such embodiment the nucleic acid encodes a bacterial enzyme that comprises an amino acid sequence of SEQ ID NO:2. In another embodiment the nucleic acid encodes a bacterial enzyme that comprises the amino acid sequence of SEQ ID NO:2 comprising a conservative amino acid substitution. In related embodiments, the nucleic acid encodes a bacterial enzyme that comprises the amino acid sequence of SEQ ID NO:4 or the amino acid sequence of SEQ ID NO:4 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:6, or the amino acid sequence of SEQ ID NO:6 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:10, or the amino acid sequence of SEQ ID NO:10 comprising a conservative amino acid substitution, or the

amino acid sequence of SEQ ID NO:12, or the amino acid sequence of SEQ ID NO:12 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:14, or the amino acid sequence of SEQ ID NO:14 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:16, or the amino acid sequence of SEQ ID NO:16 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:18, or the amino acid sequence of SEQ ID NO:18 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:20, or the amino acid sequence of SEQ ID NO:20 comprising a conservative amino acid substitution.

In other embodiments the nucleic acid encodes a bacterial enzyme that comprises the amino acid sequence of SEQ ID NO:28 or the amino acid sequence of SEQ ID NO:28 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:30, or the amino acid sequence of SEQ ID NO:30 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:34, or the amino acid sequence of SEQ ID NO:34 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:38, or the amino acid sequence of SEQ ID NO:38 comprising a conservative amino acid substitution.

The present invention further provides a nucleic acid encoding a bacterial enzyme that comprises an amino acid sequence of SEQ ID NO:52. In another embodiment the nucleic acid encodes a bacterial enzyme that comprises the amino acid sequence of SEQ ID NO:52 comprising a conservative amino acid substitution. In related embodiments, the nucleic acid encodes a bacterial enzyme that comprises the amino acid sequence of SEQ ID NO:54 or the amino acid sequence of SEQ ID NO:54 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:56, or the amino acid sequence of SEQ ID NO:56 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:50, or the amino acid sequence of SEQ ID NO:50 comprising a conservative amino acid substitution.

In a particular embodiment the isolated nucleic acid comprises the nucleotide

sequence of SEQ ID NO:1. In related embodiments the isolated nucleic acid comprises the nucleotide sequence of SEQ ID NO:3, or the nucleotide sequence of SEQ ID NO:5, or the nucleotide sequence of SEQ ID NO:9, or the nucleotide sequence of SEQ ID NO:11, or the nucleotide sequence of SEQ ID NO:13, or the nucleotide sequence of SEQ ID NO:15, or the nucleotide sequence of SEQ ID NO:17, or the nucleotide sequence of SEQ ID NO:19.

Still other related embodiments comprise the nucleotide sequence of SEQ ID NO:27, or the nucleotide sequence of SEQ ID NO:29, or the nucleotide sequence of SEQ ID NO:33, or the nucleotide sequence of SEQ ID NO:37.

In a particular embodiment the isolated nucleic acid comprises the nucleotide sequence of SEQ ID NO:51. In related embodiments the isolated nucleic acid comprises the nucleotide sequence of SEQ ID NO:53, or the nucleotide sequence of SEQ ID NO:55, or the nucleotide sequence of SEQ ID NO:49.

The polypeptides encoded by all of the novel nucleic acids disclosed above are also part of the present invention.

The present invention also includes an isolated nucleic acid that hybridizes under standard hybridization conditions to a nucleic acid (*e.g.*, a cDNA) comprising one or more of the nucleotide sequences of the present invention. In a preferred embodiment the isolated nucleic acid hybridizes to the nucleotide sequence of SEQ ID NO:1. In another preferred embodiment the isolated nucleic acid hybridizes to the nucleotide sequence of SEQ ID NO:51. In related embodiments, the isolated nucleic acid hybridizes to the nucleotide sequence of SEQ ID NO:3, and/or the nucleotide sequence of SEQ ID NO:5, and/or the nucleotide sequence of SEQ ID NO:9, and/or the nucleotide sequence of SEQ ID NO:11, and/or the nucleotide sequence of SEQ ID NO:13, and/or the nucleotide sequence of SEQ ID NO:15, and/or the nucleotide sequence of SEQ ID NO:17, and/or the nucleotide sequence of SEQ ID NO:19. In still other related embodiments the isolated nucleic acid hybridizes to the nucleotide sequence of the nucleotide sequence of SEQ ID NO:53, and/or the nucleotide

sequence of SEQ ID NO:55, and/or the nucleotide sequence of SEQ ID NO:49.

Such nucleic acids include those that can act as probes or primers for one or more of the nucleotide sequences of the present invention. The polypeptides encoded by the novel nucleic acids that hybridize to the nucleic acids described above are also part of the present invention.

The present invention further provides a recombinant DNA molecule that comprises an isolated nucleic acid of the present invention, as described above, with or without a heterologous nucleotide sequence. Such a recombinant DNA molecule can be operatively linked to an expression control sequence and can be part of an expression vector. The present invention further provides a cell that comprises such an expression vector. The cell can be either a eukaryotic or preferably a prokaryotic cell. The present invention further provides a method of expressing a recombinant polypeptide of the present invention or fragment thereof in this cell. One such method comprises culturing the cell in an appropriate cell culture medium under conditions that provide for expression of the polypeptide by the cell. Preferably the recombinant polypeptide comprises the amino acid sequence of SEQ ID NO:45, can bind a flavin prosthetic group, and can act enzymatically as an enoyl reductase. In an alternative embodiment the recombinant polypeptide comprises the amino acid sequence of SEQ ID NO:57, does not contain a flavin prosthetic group, and can act enzymatically as an enoyl reductase. In a preferred embodiment the method comprises the step of purifying the recombinant polypeptide. The recombinant polypeptide purified by the method is also part of the present invention.

The present invention further provides a nucleic acid that encodes a polypeptide that binds a flavin prosthetic group, has enoyl reductase activity and has at least 30%, preferably 60%, more preferably 75%, even more preferably 90% and most preferably 95% amino acid identity with a bacterial enzyme comprising the amino acid sequence of SEQ ID NO:2. In a preferred embodiment the nucleic acid encodes a FabK. In related embodiments, the nucleic acid encodes a polypeptide that binds a flavin prosthetic group, has enoyl reductase activity and has at least 60%, preferably

80%, and more preferably 90% amino acid identity with a bacterial enzyme comprising the amino acid sequence of SEQ ID NO:4, and/or the amino acid sequence of SEQ ID NO:6, and/or the amino acid sequence of SEQ ID NO:10, and/or the amino acid sequence of SEQ ID NO:12, and/or the amino acid sequence of SEQ ID NO:14, and/or the amino acid sequence of SEQ ID NO:16, and/or the amino acid sequence of SEQ ID NO:18, and/or the amino acid sequence of SEQ ID NO:20. Again in preferred embodiments the nucleic acid encodes a FabK. The polypeptides encoded by the nucleic acids described above are also part of the present invention.

The present invention also provides a nucleic acid that encodes a polypeptide that does not contain a flavin prosthetic group, has enoyl reductase activity and has at least 40%, preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95% amino acid identity with a bacterial enzyme comprising the amino acid sequence of SEQ ID NO:52. The polypeptides encoded by the nucleic acids described above are also part of the present invention.

The present invention also provides a nucleic acid that encodes a polypeptide that binds a flavin prosthetic group, has enoyl reductase activity and that comprises at least 8, preferably12 and more preferably 16 consecutive amino acids of a bacterial enzyme that has an amino acid sequence of SEQ ID NO:2. In a preferred embodiment the nucleic acid encodes a FabK. In related embodiments, the nucleic acid encodes a polypeptide that binds a flavin prosthetic group, has enoyl reductase activity and comprises at least 8, preferably12 and more preferably 16 consecutive amino acids of a bacterial enzyme that has an amino acid sequence of SEQ ID NO:4, and/or the amino acid sequence of SEQ ID NO:6, and/or the amino acid sequence of SEQ ID NO:10, and/or the amino acid sequence of SEQ ID NO:12, and/or the amino acid sequence of SEQ ID NO:14, and/or the amino acid sequence of SEQ ID NO:16, and/or the amino acid sequence of SEQ ID NO:18, and/or the amino acid sequence of SEQ ID NO:20. In preferred embodiments the nucleic acid encodes a FabK. The polypeptides encoded by the nucleic acids described above are also part of the present invention.

The present invention further provides a nucleic acid that encodes a polypeptide that does not contain a flavin prosthetic group, has enoyl reductase activity and that comprises at least 8, preferably12 and more preferably 16 consecutive amino acids of a bacterial enzyme that has an amino acid sequence of SEQ ID NO:52. In a preferred embodiment the nucleic acid encodes a FabL. In related embodiments, the nucleic acid encodes a polypeptide that does not contain a flavin prosthetic group, has enoyl reductase activity and comprises at least 8, preferably12 and more preferably 16 consecutive amino acids of a bacterial enzyme that has an amino acid sequence of SEQ ID NO:54, and/or the amino acid sequence of SEQ ID NO:56, and/or the amino acid sequence of SEQ ID NO:50. The polypeptides encoded by the nucleic acids described above are also part of the present invention.

The present invention further provides fragments of the polypeptides of the present invention and fusion proteins/peptides including chimeric proteins and intein fusion proteins/peptides. The fusion proteins/peptides can comprise any of the polypeptides of the present invention including the fragments of the polypeptides. Such fragments include antigenic fragments, proteolytic fragments, such as peptides prepared by treatment with a protease *e.g.*, trypsin, active fragments that retain enoyl reductase activity, and peptides comprising at least 5, preferably 12 and more preferably 20 consecutive amino acids of a bacterial enzyme that has the amino acid sequence of SEQ ID NO:2 and/or the amino acid sequence of SEQ ID NO:4, and/or the amino acid sequence of SEQ ID NO:6, and/or the amino acid sequence of SEQ ID NO:10, and/or the amino acid sequence of SEQ ID NO:12, and/or the amino acid sequence of SEQ ID NO:14, and/or the amino acid sequence of SEQ ID NO:16, and/or the amino acid sequence of SEQ ID NO:18, and/or the amino acid sequence of SEQ ID NO:20. In a particular embodiment, the antigenic fragment comprises the amino acid sequence of SEQ ID NO:46 or SEQ ID NO:46 comprising a conservative amino acid substitution.

In a related embodiment such fragments comprise at least 5, preferably 12 and more preferably 20 consecutive amino acids of a bacterial enzyme that has the amino acid sequence has the amino acid sequence of SEQ ID NO:52 and/or the amino acid

sequence of SEQ ID NO:54, and/or the amino acid sequence of SEQ ID NO:56, and/or the amino acid sequence of SEQ ID NO:50. In a particular embodiment, the antigenic fragment comprises the amino acid sequence of SEQ ID NO:58 or SEQ ID NO:58 comprising a conservative amino acid substitution.

The present invention also provides fragments and fusion proteins/peptides as defined above for the enoyl reductases having the amino acid sequence of SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:34, and SEQ ID NO:38.

In addition, the present invention provides proteins and fragments and fusion proteins/peptides as defined above having the amino acid sequences of SEQ ID NO:8, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:32, SEQ ID NO:36 and SEQ ID NO:48.

The present invention also provides immunogenic compositions and vaccines. In a particular embodiment the vaccine comprises an antigenic fragment of the present invention. Antibodies to the enoyl reductases and antigenic fragments of the present invention are also included. Such antibodies can be monoclonal antibodies, and/or chimeric antibodies or polyclonal antibodies. The present invention further provides an immortal cell line that produces a monoclonal antibody of the present invention. In a particular embodiment, the monoclonal antibody is raised against a polypeptide or fragment thereof comprising SEQ ID NO:46. In another embodiment, the monoclonal antibody is raised against a polypeptide or fragment thereof comprising SEQ ID NO:58.

The present invention further provides methods for identifying agents that can modulate the enzymatic activity of an enoyl reductase of the present invention. One such embodiment comprises measuring the enzymatic activity of an enoyl reductase or active fragment thereof in the presence and absence of a compound. The compound is identified as an agent that modulates the enzymatic activity of an enoyl reductase when the enzymatic activity measured is different in the presence of the compound relative to in the absence of the compound. In a preferred embodiment of this type, the enoyl reductase comprises the amino acid sequence of SEQ ID NO:45

and contains a flavin prosthetic group. In another preferred embodiment of this type, the enoyl reductase comprises the amino acid sequence of SEQ ID NO:57 and does not contain a flavin prosthetic group. In a particular embodiment, the enzymatic activity is lower in the presence of the compound relative to in the absence of the compound. In this case the compound is identified as an inhibitor of the enoyl reductase. In another embodiment of this type, the enzymatic activity is higher in the presence of the compound relative to in the absence of the compound. In this case the compound is identified as an agonist of the enoyl reductase. In one particular embodiment, the enoyl reductase is a FabK. In another particular embodiment, the enoyl reductase is a FabL.

The present invention further provides methods for identifying an agent that can bind to an enoyl reductase. One such embodiment comprises contacting an enoyl reductase or active fragment thereof with a compound and determining whether the compound binds to the enoyl reductase. A compound is identified as an agent that can bind the enoyl reductase if the compound binds to the enoyl reductase. In a preferred embodiment of this type, the enoyl reductase comprises the amino acid sequence of SEQ ID NO:45 and contains a flavin prosthetic group. In another preferred embodiment of this type, the enoyl reductase comprises the amino acid sequence of SEQ ID NO:57 and does not contain a flavin prosthetic group.

In the *in vitro* studies involving the enoyl reductases of the present invention, the enoyl reductase preferably has the amino acid sequence of SEQ ID NO:2. However, in other embodiments of the present invention, the enoyl reductase has the amino acid sequence of SEQ ID NO:2 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:4, or the amino acid sequence of SEQ ID NO:4 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:6,or the amino acid sequence of SEQ ID NO:6 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:10, or the amino acid sequence of SEQ ID NO:10 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:12, or the amino acid sequence of SEQ ID NO:12 comprising a conservative amino acid substitution, or the

amino acid sequence of SEQ ID NO:14, or the amino acid sequence of SEQ ID NO:14 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:16, or the amino acid sequence of SEQ ID NO:16 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:18, or the amino acid sequence of SEQ ID NO:18 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:20, or the amino acid sequence of SEQ ID NO:20 comprising a conservative amino acid substitution SEQ ID NO:22.

Similarly, the enoyl reductase can comprise the amino acid sequence of SEQ ID NO:52. In another embodiment the enoyl reductase comprises the amino acid sequence of SEQ ID NO:52 comprising a conservative amino acid substitution. In related embodiments, the enoyl reductase comprises the amino acid sequence of SEQ ID NO:54 or the amino acid sequence of SEQ ID NO:54 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:56, or the amino acid sequence of SEQ ID NO:56 comprising a conservative amino acid substitution, or the amino acid sequence of SEQ ID NO:50, or the amino acid sequence of SEQ ID NO:50 comprising a conservative amino acid substitution.

As mentioned above, fusion proteins/peptides and/or fragments, and preferably active fragments of the enoyl reductases can also be used.

The present invention further provides methods for identifying a drug that inhibits bacterial growth. One such embodiment comprises administering an agent that is suspected of inhibiting an enoyl reductase of the present invention to a bacterial cell and then determining the growth of the cell. An agent that inhibits the growth of the cell relative to the growth in the absence of the agent is identified as a drug that inhibits bacterial growth. In a preferred embodiment of this type, the enoyl reductase comprises the amino acid sequence of SEQ ID NO:45 and contains a flavin prosthetic group. In a more preferred embodiment, the enoyl reductase is a FabK. Alternatively, the enoyl reductase comprises the amino acid sequence of SEQ ID NO:57 and does not contain a flavin prosthetic group. In a more preferred

embodiment of this type, the enoyl reductase is a FabL.

As should be readily understood, any of the methods described above can be performed either alone, or in tandem including the combination of two or more of the above-described methods. For example, an agent could be first tested for binding, then tested for inhibiting the enoyl reductase. An agent that both binds the enoyl reductase and inhibits the enoyl reductase activity could then be tested to determine if it also inhibited bacterial cell growth. Further studies could be performed in an animal model to determine if the agent was effective in either preventing or treating a bacterial infection. An agent found to be effective in an animal model could then be used in a clinical study. Thus the present invention further provides the agents and drugs identified by the methods of the present invention and the corresponding pharmaceutical compositions, which can further comprise a pharmaceutically acceptable carrier.

Accordingly, it is a principal object of the present invention to provide novel enoyl reductases. Such enzymes can used as targets in drug discovery including for high throughput screening and/or rational drug design.

It is a further object of the present invention to provide methods of using these enoyl reductases to identify agents that will act against bacterial infections.

It is a further object of the present invention to provide antibacterial agents obtained by the methods of the present invention.

It is a further object of the present invention to provide structural and enzymatic characteristics and properties of the enoyl reductases, including their nucleic acid and amino acid sequences.

It is a further object of the present invention to provide an antibody specific for FabK.

It is a further object of the present invention to provide an antibody specific for FabL.

It is a further object of the present invention to provide an immunogenic composition comprising a FabK, or an antigenic fragment of FabK.

It is a further object of the present invention to provide a vaccine comprising a nucleic acid encoding a FabK or an antigenic fragment of a FabK.

It is a further object of the present invention to provide an immunogenic composition comprising a FabK, or an antigenic fragment of FabL

It is a further object of the present invention to provide a vaccine comprising a nucleic acid encoding a FabK or an antigenic fragment of a FabL.

It is a further object of the present invention to provide a method of producing an enoyl reductase of the present invention, including through modification or fragmentation of an enoyl reductase through recombinant technology.

It is a further object of the present invention to provide a method of performing rational drug design with the use of an enoyl reductase of the present invention.

These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the *fab* gene cluster in *S. pnemoniae* and the predicted FabK protein sequence. Figure 1A shows the *fab* gene cluster which was defined using the primary sequence of the *E. coli* proteins required for fatty acid synthesis to search the *S. pneumoniae* genome for homologous proteins in the TIGR database. The genes corresponding to *fabH, acpP, fabG, fabD, fabF, fabZ* and *accABCD* were clustered on a 10 kbp fragment. A FabI homolog was not present in the *S. pneumoniae* genome, but there was an unidentified open reading frame within the *fab* cluster that is designated as *fabK*. Figure 1B shows the deduced protein sequences of FabK. The

predicted FabK protein sequence from *S. pneumoniae* is aligned with the predicted sequences of FabK proteins from a representative group of bacteria. Similar amino acids among all six proteins are boxed based on the following similarity groups: P, A, G, S, T; Q, N, E, D; H, K, R; C; V, L, I, M; and F, Y, W. The region of the proteins containing a consensus binding site for FAD is indicated.

Figures 2A-2D show the purification and characterization of FabK. Figure 2A shows a gel with the isolated FabK possessing an amino terminal His-tag. The FabK was expressed in *E. coli* strain BL21-Codon Plus-(DE3)-R1L (Stratagene) grown in LB medium supplemented with riboflavin (0.5 g/L). His-tag FabK was purified by metal chelation affinity chromatography as described in the Example, below. The purified protein migrated at 36 kDa when subjected to SDS gel electrophoresis, corresponding to the predicted molecular weight of the protein plus the His-tag. The purified protein solution was bright yellow, and the UV-Vis spectrum showed absorption maxima at 270, 350 and 450 nm (*solid line,* Figure 2B). The absorption at 350 and 450 nm is characteristic of flavin. An aliquot of FabK was boiled for 30 min. The denatured protein that was removed had an FabK:FAD ratio of 0.8 that was calculated using an extinction coefficients of 20.3 at 280 nm for FabK and 11.3 at 450 nm for FAD. Fluoresence spectroscopy (*inset*, Figure 2B) of the purified FabK protein (*solid line,* Figure 2B) excitated at 417 nm showed an emmission maxima at 535 nm, which was the same as free FAD (*dashed line,* Figure 2B). The cofactor was identified as FAD by thin-layer chromatography on silica gel 60 layers developed with 5% Na_2HPO_4. A coupled enzyme system was employed to assay FabK by combining the purified *E. coli* proteins required to reconstitute a cycle of fatty acid synthesis with the exception of the FabI enoyl-ACP reductase. Reaction mixtures were first incubated to generate *trans*-2-butenoyl-ACP and then the indicated amounts of FabK were added as described in the Example below. Products were separated by conformationally sensitive gel electrophoresis and the bands visualized and quantitated using a Phosphorimager (Figure 2C). Figure 2D is a plot of the rate of formation of butyryl-ACP as a function of FabK concentration derived from the data in Figure 2C.

Figure 3 shows the alignment of two Fab L proteins (from *Bacillus subtilis*, and

Helicobacter pylori) with FabI from *E. coli*. "+" indicates key residues in *E. coli*. FabI. Similar amino acids among all six proteins are boxed based on the following similarity groups:

P, A, G, S, T; Q, N, E, D; H, K, R; C; V, L, I, M; and F, Y, W.

DETAILED DESCRIPTION OF THE INVENTION

The enoyl-acyl carrier protein (ACP) reductase (FabI) of *Escherichia coli* catalyzes the final step of each round in fatty acid elongation. Because it is essential in bacterial metabolism, it is considered an important the target for anti-bacterials. One such antibacterial is triclosan, which is commonly found in antibacterial hand soaps and related products. The present invention provides a new bacterial enzyme that catalyzes the identical reaction that FabI catalyzes. This new bacterial enzyme has been named FabK. In addition, the present invention provides a number of novel related enzymes that can also act as enoyl reductases.

As disclosed herein, many Gram-positive bacteria express a unique enoyl reductase that has been disclosed herein and named, FabK. One such *fabK* gene is located within the fatty acid biosynthetic gene cluster of *Streptococcus pneumoniae* and encodes a flavoprotein that catalyzes the NADH-dependent reduction of enoyl-ACPs. FabI is shown herein to be the only target for triclosan in *E. coli* because *fabK* expression rescues the temperature-sensitive growth phenotype of an *E. coli fabI*(Ts) mutant and confers complete triclosan resistance. In addition, a second unique enoyl reductase has been found as disclosed herein, and is named FabL (*see* Table 3, below). The discovery of these new enoyl-ACP reductases reveal a unique mechanism for enoyl-ACP reduction that could be exploited for the development of novel antibacterial therapies.

Thus the discovery of two new families of enoyl-ACP reductases both having a significantly different structure from the known FabI proteins has important implications for antibacterial drug development. Since FabK and FabL catalyze the same reaction as FabI, inhibitors of FabK and/or FabL would be an effective

bactericide against bacteria and other microorganisms that express FabK and/or FabL rather than FabI. Indeed, microorganisms that express FabK and/or FabL rather than FabI should be refractory to specific FabI inhibitors. Conversely, the development of selective FabK and/or FabL inhibitors would be an effective strategy against several important pathogens, such as *Streptococci* and *Clostridia*, but would not be effective against strains expressing FabI. Clearly, organisms like the *Pseudomonads* and *Enterococci* that contain both a FabI and FabK would require a combination therapy of enoyl-ACP reductase inhibitors to block cell growth. Thus, enoyl-ACP reductase based therapies can be tailored for specific pathogens based on their expression of FabI and/or FabK and/or FabL.

Therefore, if appearing herein, the following terms shall have the definitions set out below:

As used herein a "FabK" is an enzyme that comprises the consensus sequence of SEQ ID NO:45 and a flavin, and is capable of catalyzing the NAD(P)H-dependent reduction of an enoyl-ACP. One example of a particular FabK is an enoyl reductase from *S. pneumoniae* having the amino acid sequence of SEQ ID NO:2.

As used herein a "FabL" is an enzyme that comprises the consensus sequence of SEQ ID NO:57, does not contain a flavin, and is capable of catalyzing the reduction of an enoyl-ACP. One example of a particular FabL is an enoyl reductase from *Campylobacter jejuni* having the amino acid sequence of SEQ ID NO:52.

As used herein an "active fragment" of a polypeptide has an amino acid sequence that corresponds to that of the corresponding full-length protein except the active fragment has at least one less amino acid than the corresponding full-length protein; furthermore an "active fragment" of an enoyl reductase of the present invention has at least 20% of the enoyl reductase activity of the corresponding full-length protein when determined under at least one set of conditions in which the full-length protein has enoyl reductase activity.

As used herein a protein or fragment thereof has "enoyl reductase activity" when it has the ability to reduce a *trans*-2-enoyl thioester to an acyl-thioester.

As used herein a "polypeptide" is used interchangably with the term "protein" and denotes a polymer comprising two or more amino acids connected by peptide bonds. Preferably, a polypeptide is further distinguished from a "peptide" with a peptide comprising about twenty or less amino acids, and a polypeptide or protein comprising more than about twenty amino acids.

As used herein the terms "fusion protein" and "fusion peptide" are used interchangeably and encompass "chimeric proteins and/or chimeric peptides" and fusion "intein proteins/peptides". A fusion protein comprises at least a portion of an enoyl reductase of the present invention joined *via* a peptide bond to at least a portion of another protein or peptide including a second enoyl reductase in a chimeric fusion protein. In a particular embodiment the portion of the enoyl reductase is antigenic. For example fusion proteins can comprise a marker protein or peptide, or a protein or peptide that aids in the isolation and/or purification of an enoyl reductase of the present invention.

As used herein the term "approximately" is used interchangeably with the term "about" and signifies that a value is within ten percent of the indicated value *i.e.*, a protein containing "approximately" 500 amino acid residues can contain between 450 and 550 amino acid residues.

As used herein a "small organic molecule" is an organic compound [or organic compound complexed with an inorganic compound (*e.g.*, metal)] that has a molecular weight of less than 3 Kilodaltons.

As used herein the term "binds to" is meant to include all such specific interactions that result in two or more molecules showing a preference for one another relative to some third molecule. This includes processes such as covalent, ionic, hydrophobic and hydrogen bonding but does not include non-specific associations such as solvent

preferences.

A "vector" is a replicon, such as a plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control.

A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA can encode a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

A cell has been "transfected" or transformed by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transduced" by exogenous or heterologous DNA when the exogenous or heterologous DNA is introduced by a viral vector.

A "heterologous nucleotide sequence" as used herein is a nucleotide sequence that is added to a nucleotide sequence of the present invention by recombinant methods to form a nucleic acid which is not naturally formed in nature. Such nucleic acids can encode fusion (e.g. chimeric) proteins. Thus the heterologous nucleotide sequence can encode peptides and/or proteins which contain regulatory and/or structural properties. In another such embodiment the heterologous nucleotide sequence can encode a protein or peptide that functions as a means of detecting the protein or peptide encoded by the nucleotide sequence of the present invention after the recombinant nucleic acid is expressed. In still another embodiment the heterologous nucleotide sequence can function as a means of detecting a nucleotide sequence of the present invention. A heterologous nucleotide sequence can comprise non-coding sequences including restriction sites, regulatory sites, promoters and the like.

A "heterologous" region of the DNA construct is an identifiable segment of DNA

within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (*e.g.*, a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. When referring to a nucleic acid that is DNA, and more specifically a DNA having a particular nucleotide sequence, *i.e.*, SEQ ID NO:1, both the "sense" strand and the complementary "antisense" strand are intended to be included. Thus a nucleic acid that is hybridizable to SEQ ID NO:1, for example, can be either hybridizable to the "sense" strand of SEQ ID NO:1, which is particularly listed in the SEQUENCE LISTING, or to the "antisense" strand which can be readily determined from that SEQUENCE LISTING.

The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary form. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant

DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength [*see* Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.*, 1989"]. The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS. Moderate stringency hybridization conditions correspond to a higher T_m, *e.g.*, 40% formamide, with 5x or 6x SSC. High stringency hybridization conditions correspond to the highest T_m, *e.g.*, 50% formamide, 5x or 6x SSC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived [*see* Sambrook *et al.*, *supra*, 9.50-10.51]. For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity [*see* Sambrook *et al.*, *supra*, 11.7-11.8]. Preferably a minimum length for a hybridizable nucleic acid is at least about 12 nucleotides; preferably at least about 18 nucleotides; and more preferably the length is at least about 27 nucleotides; and most preferably at least about 36 nucleotides.

In a specific embodiment, the term "standard hybridization conditions" refers to a T_m of 55°C, and utilizes conditions as set forth above e.g., 5X SSC. In a preferred embodiment, the T_m is 60°C; in a more preferred embodiment, the T_m is 65°C.

"Homologous recombination" refers to the insertion of a foreign DNA sequence of a vector in a chromosome. Preferably, the vector targets a specific chromosomal site for homologous recombination. For specific homologous recombination, the vector will contain sufficiently long regions of homology to sequences of the chromosome to allow complementary binding and incorporation of the vector into the chromosome. Longer regions of homology, and greater degrees of sequence similarity, may increase the efficiency of homologous recombination.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

"Transcriptional and translational control sequences" are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3′ terminus by the transcription initiation site and extends upstream (5′

direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence.

A "signal sequence" is included at the beginning of the coding sequence of a protein to be expressed on the surface of a cell. This sequence encodes a signal peptide, N-terminal to the mature polypeptide, that directs the host cell to translocate the polypeptide. The term "translocation signal sequence" is used herein to refer to this sort of signal sequence. Translocation signal sequences can be found associated with a variety of proteins native to eucaryotes and prokaryotes, and are often functional in both types of organisms.

As used herein, the term "sequence homology" in all its grammatical forms refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, etc.) [Reeck *et al.*, *Cell* **50**:667 (1987)].

As used herein, the term "ortholog" refers to the relationship between proteins that have a common evolutionary origin and differ because they originate from different species or strain. For example, *P. aeruginosa* FabK is an ortholog of *S. pneumoniae* FabK.

The term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins

that do not share a common evolutionary origin (*see* Reeck *et al.*, *supra*). However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and not necessarily a common evolutionary origin.

In a specific embodiment, two highly homologous DNA sequences can be identified by their own homology, or the homology of the amino acids they encode. Such comparison of the sequences can be performed using standard software available in sequence data banks. In a particular embodiment two highly homologous DNA sequences encode amino acid sequences having 30%, preferably 50%, more preferably 70% and even more preferably 80% identity. More particularly, two highly homologous amino acid sequences have 30%, preferably 50%, more preferably 70% and even more preferably 80% identity.

Alternatively, two highly homologous DNA sequences can be identified by Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook *et al.*, *supra*; DNA Cloning, Vols. I & II, *infra*; Nucleic Acid Hybridization, *infra*.

As used herein an amino acid sequence is 100% "homologous" to a second amino acid sequence if the two amino acid sequences are identical, and/or differ only by neutral or conservative substitutions as defined below. Accordingly, an amino acid sequence is 50% "homologous" to a second amino acid sequence if 50% of the two amino acid sequences are identical, and/or differ only by neutral or conservative substitutions.

As used herein, DNA and protein sequence percent identity can be determined using software such as MacVector 6.0.1, Oxford Molecular Group PLC (1996) and the Clustal W algorithm with the alignment default parameters, and default parameters for identity. These commercially available programs can also be used to determine sequence similarity using the same or analogous default parameters.

The term "corresponding to" is used herein to refer to similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic polypeptide contains at least about 5, and preferably at least about 10, amino acids. An antigenic portion of a molecule can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier molecule for immunization. A molecule that is antigenic need not be itself immunogenic, *i.e.*, capable of eliciting an immune response without a carrier.

Enoyl Reductases and Fragments Thereof

The present invention provides isolated and/or recombinant unicellular enoyl reductases and fragments thereof. In a preferred embodiment the unicellular enoyl reductase is a bacterial FabK protein or a FabL protein. FabK and FabL are enoyl-acyl carrier protein reductases that play an essential role in fatty acid synthesis for specific microorganisms and plants by catalyzing the last step in each round of elongation in the type II fatty acid synthase pathway.

The enoyl reductases of the present invention can be from any species, but are preferably from a plant or unicellular organism. Bacterial species of origin for the enoyl reductases include *S . pneumoniae*, *S. mutans, S. pyogenes, E. faecalis, C. acetobutylicum, C. difficile, P. gingivalis, Ca. cresentus, Ps. aeruginosa, Mycobacterium tuberculosis*, *H. pylori and T. martima.* Examples of nucleic acids and amino acid sequences encoding such enoyl reductases are included in Table 3, below.

In a preferred embodiment the enoyl reducatase is a FabK protein from *S. pneumoniae*. In another embodiment the FabK is a protein encoded by a nucleotide sequence that is hybridizable with the complementary strand of the coding sequence of SEQ ID NO: 1 under standard, and/or stringent conditions. In yet another embodiment the *S. pneumoniae* FabK has an amino acid sequence of SEQ ID NO:2. In still another embodiment the bacterial enoyl reductase is from *S. mutans* and is encoded by a nucleotide sequence having the coding sequence of SEQ ID NO:3. In yet another embodiment the bacterial enoyl reductase has an amino acid sequence of SEQ ID NO:4. In another preferred embodiment the enoyl reducatase is a FabL from *Campylobacter jejuni* having the amino acid sequence of SEQ ID NO:52. The enoyl reductases of the present invention may be used for many purposes including in assays to identify novel drugs such as new antibiotics, and the like, and/or can be used in protein structural and mechanistic studies.

Modified enoyl reductases: The present invention also provides "modified enoyl reductases" *i.e.*, enoyl reductase that are tagged proteins, labeled proteins, intein fusion proteins, and fusion proteins such as a chimeric protein and the like. Such enoyl reductases may be used for example as antigens or as marker proteins. In a particular embodiment of this type, the fusion protein comprises an enoyl reductase that is a FabK protein (or FabK fragment thereof) having an amino acid sequence of SEQ ID NO:2 or SEQ ID NO: 2 comprising one or more conservative amino acid substitutions. In another embodiment of this type, the fusion protein comprises an enoyl reductase or (enoyl reductase fragment thereof) having an amino acid sequence of SEQ ID NO: 4 or SEQ ID NO:4 comprising one or more conservative amino acid substitutions. Preferably such enoyl reductases or fragments thereof retain their catalytic activity. One particular use of the enoyl reductase fusion proteins of the present invention is for the production of the enoyl reductase-antibodies of the present invention.

An enoyl reductase fusion protein can comprise at least a portion of a non-enoyl reductase protein joined *via* a peptide bond to at least a portion of an enoyl reductase polypeptide. Alternatively, a chimeric enoyl reductase can be constructed comprising

portions of two or more different enoyl reductases. In preferred embodiments a portion of the enoyl reductase is functional, *i.e.*, retaining its catalytic activity. The non-enoyl reductase sequences can be amino- or carboxy-terminal to the enoyl reductase sequences. More preferably, for stable expression of an enoyl reductase fusion protein, such as a FabK fusion protein, the portion of the non-FabK fusion protein (or tag such as a His-tag exemplified below), is joined *via* a peptide bond to the amino terminus of the FabK protein. A recombinant DNA molecule encoding such a fusion protein comprises a sequence encoding at least a portion of a non-enoyl reductase protein joined in-frame to the enoyl reductase coding sequence, and can encode a cleavage site for a specific protease, *e.g.*, thrombin or Factor Xa, preferably at or close to the enoyl reductase-non-enoyl reductase juncture. In a specific embodiment, the fusion protein is expressed in *Escherichia coli*. Such a fusion protein can be used to isolate the enoyl reductases of the present invention, through the use of an affinity column which is specific for the protein and/or tag fused to the enoyl reductase, such as from *S. pneumoniae* as exemplified below. The purified FabK for example, may then be released from the fusion protein through the use of a proteolytic enzyme and a cleavage site such as has been referred to above.

In one such embodiment, a chimeric enoyl reductase can be prepared, *e.g.*, a glutathione-S-transferase (GST) fusion protein, a maltose-binding (MBP) protein fusion protein, or a poly-histidine-tagged fusion protein, for expression in any cell, or alternatively in a cell-free system. Expression of an enoyl reductase, such as a FabK, as a fusion protein can facilitate stable expression, or allow for purification based on the properties of the fusion partner. For example, GST binds glutathione conjugated to a solid support matrix, MBP binds to a maltose matrix, and poly-histidine chelates to a Ni-chelation support matrix, as exemplified below. The fusion protein can be eluted from the specific matrix with appropriate buffers, or by treating with a protease specific for a cleavage site usually engineered between the enoyl reductase and the fusion partner (*e.g.*, GST, MBP, or poly-His) as described above. Alternatively the chimeric enoyl reductase protein may contain the green fluorescent protein, and be used to determine the intracellular localization of the enoyl reductase in the cell.

Genes Encoding Enoyl Reductases

The present invention contemplates isolation of a gene encoding an enoyl reductase of the present invention, such as a FabK or a FabL, including a full length, or naturally occurring form of an enoyl reductase, and antigenic fragments thereof from any plant or microorganism, but preferably a bacterial source. Such nucleic acids may be used for designing primers for RT-PCR, and for making probes that are useful for determining the expression of a *fabK* or *fabL* messenger RNA, for example. Similarly, such nucleic acids can be used to determine the expression of the *fabK* or *fabL* messenger RNA by Northern Blot analysis, RNA protection assays and the like. As used herein, the term "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids. Therefore, the present invention provides the primary structure of genes encoding enoyl reductases such as the *S. pneumoniae* FabK protein exemplified below.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. [See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.*, 1989"); *DNA Cloning: A Practical Approach,* Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait *ed.*, 1984); *Nucleic Acid Hybridization* B.D. (Hames & S.J. Higgins eds., 1985); *Transcription And Translation* B.D. (Hames & S.J. Higgins, eds., 1984); *Animal Cell Culture* R.I. (Freshney, ed.,1986); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994)].

A gene encoding an enoyl reductase of the present invention whether genomic DNA or cDNA, can be isolated from any source, preferably from a bacterial source. Thus, in view and in conjunction with the present teachings, methods well known in the art, as described above can be used for obtaining enoyl reductase genes from any source (*see, e.g.*, Sambrook *et al.*, 1989, *supra*). These methods can be supplemented and/or

used in the alternative with the use of nucleic acid and/or protein databases (either complete or partially complete) to identify new *FabK* orthologues for example. Such identification can then lead to the subsequent isolation of the gene and/or protein as exemplified below.

Accordingly, any plant cell and/or microorganism can potentially serve as the nucleic acid source for the molecular cloning of an *enoyl reductase* gene. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), and preferably is obtained from an appropriate cDNA library, by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell (See, for example, Sambrook *et al.*, 1989, *supra*; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II). Clones derived from higher genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will not contain intron sequences. Whatever the source, the gene can be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments can be generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing the desired enoyl reductase gene may be accomplished in a number of ways. For example, the generated DNA fragments may be screened by nucleic acid hybridization to a labeled probe of the present invention [Benton and Davis, *Science* **196**:180 (1977); Grunstein and Hogness, *Proc. Natl. Acad. Sci. U.S.A.* **72**:3961 (1975)]. For example, a set of oligonucleotides corresponding to the sequence information provided by the present invention can be prepared and used as probes for

DNA encoding the FabK exemplified below (*e.g.*, in combination with a poly-T primer for RT-PCR). Preferably, a probe is selected that is highly unique to the FabK of the invention. Those DNA fragments with substantial homology to the probe will hybridize. As noted above, the greater the degree of homology, the more stringent hybridization conditions can be used.

Further selection can be carried out on the basis of the properties of the gene, *e.g.*, if the gene encodes a protein product having the activity, isoelectric point, electrophoretic properties, amino acid composition, or partial amino acid sequence of an enoyl reductase as disclosed herein. Thus, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that, *e.g.*, has similar or identical electrophoretic migration, isoelectric focusing or non-equilibrium pH gel electrophoresis behavior, proteolytic digestion maps, or antigenic properties as known for FabK, for example.

An enoyl reductase gene of the invention can also be identified by mRNA selection, *i.e.*, by nucleic acid hybridization followed by *in vitro* translation. In this procedure, nucleotide fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified enoyl reductase DNA, or may be synthetic oligonucleotides designed from the partial amino acid sequence information. Immunoprecipitation analysis or functional assays (*e.g.*, capable of acting as enoyl reductase as defined herein) of the *in vitro* translation products of the products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments, that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against an enoyl reductase such as FabK.

A radiolabeled enoyl reductase cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabeled mRNA or cDNA may then be used as a probe to identify homologous enoyl reductase DNA fragments from

among other genomic DNA fragments.

The present invention also relates to cloning vectors containing genes encoding analogs and derivatives of the enoyl reductases of the present invention, that have the same or homologous functional activity such as the enzymatic activity of FabK, having an amino acid sequence of SEQ ID NO:2, and in particular orthologs thereof from other species. The production and use of derivatives and analogs related to the enoyl reductases of the present invention are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, *i.e.*, capable of acting as enoyl reductase as defined herein.

Enoyl reductase derivatives can be made by altering encoding nucleotide sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Preferably, derivatives are made that have enhanced or increased functional activity, or greater antigenic specificity.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as an enoyl reductase gene of the present invention may be used in the practice of the present invention. These include but are not limited to allelic genes, homologous genes from other species, and nucleotide sequences comprising all or portions of the enoyl reductase genes which are altered by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Likewise, the enoyl reductase derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of an enoyl reductase protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution. Such alterations define the term "a conservative substitution" as used herein. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutions for an amino acid within the sequence may be selected from other

members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point.

Particularly preferred conservative substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free NH_2 can be maintained.

The amino acids also can be placed in the following similarity groups:

(1) proline, alanine, glycine, serine, and threonine;

(2) glutamine, asparagine, glutamic acid, and aspartic acid;

(3) histidine, lysine, and arginine;

(4) cysteine;

(5) valine, leucine, isoleucine, methionine; and

(6) phenylalanine, tyrosine, and tryptophan.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced at a potential site for disulfide bridges with another Cys. Pro may be introduced because of its particularly planar structure, which induces β-turns in the protein's structure.

The genes encoding enoyl reductase derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned enoyl

reductase gene sequence can be modified by any of numerous strategies known in the art (Sambrook *et al.*, 1989, *supra*). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of an enoyl reductase, care should be taken to ensure that the modified gene remains within the same translational reading frame as the enoyl reductase gene, uninterrupted by translational stop signals, in the gene region where the desired activity is encoded.

Additionally, the FabK-encoding nucleic acid sequence, for example, can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Preferably, such mutations enhance the functional and/or antigenic activity of the mutated enoyl reductase gene product. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis [Hutchinson, *et al.*, *J. Biol. Chem.* **253**:6551 (1978), Zoller and Smith, *DNA* **3**:479-488 (1984), Oliphant *et al.*, *Gene* **44**:177 (1986), and Hutchinson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**:710 (1986)] use of TAB® linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis [*see* Higuchi, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70 (1989) or as described in the Example below].

The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Examples of vectors include, but are not limited to, *E. coli*, bacteriophages such as lambda derivatives, or plasmids such as pBR322 derivatives or pUC plasmid derivatives, *e.g.*, pGEX vectors, pMal-c, pFLAG, etc. The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has

complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Recombinant molecules can be introduced into host cells via transduction, transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated. Preferably, the cloned gene is contained on a shuttle vector plasmid, which provides for expansion in a cloning cell, *e.g.*, *E. coli*, and facile purification for subsequent insertion into an appropriate expression cell line, if such is desired. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for replication in both *E. coli* and *Saccharomyces cerevisiae* by linking sequences from an *E. coli* plasmid with sequences from the yeast 2μ plasmid.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionation, can be done before insertion into the cloning vector.

The nucleotide sequence of an enoyl reductase such as the *S. pneumoniae FabK e.g.*, having the nucleotide sequence *of* SEQ ID NO:1, or more preferably the *S. pneumoniae* Fab*K* having the amino acid sequence of SEQ ID NO:2, can also be used to search for highly homologous genes from other species, including fungi using computer data bases containing full or partial nucleic acid sequences (*see* Table 3). The FabK amino acid sequence of SEQ ID NO:2, for example, can be compared with computer translated plant or fungi sequences, *e.g.*, in the appropriate databases, using software such as GCG and the BLAST search program for example. Matches with highly homologous sequences can then be obtained.

If a matched partial sequence is obtained, it can then be fully sequenced, if it has not

been already. Many methods for accomplishing this are known. One such procedure includes performing DNA sequencing reactions that can be assembled on a Beckman Biomek robotic system using standard dye-terminator chemistry, Taq polymerase and thermal cycling conditions described by the vendor [Perking Elmer/Applied Biosystems Division (PE/AB)]. Preferably sequencing is performed multiple times to ensure accuracy. Reaction products can be resolved on PE/ABD model 373 and 377 automated DNA sequencers. Contig assembly can be performed using any number of programs (*e.g.*, Gap4) and a consensus sequence can be further analyzed using the GCG suite of applications. The resulting sequence can then be used in place of, and/or in conjunction with SEQ ID NOs:1 or 2, for example, to identify other partial sequences that contain coding regions of orthologs to FabK.

Plasmids containing the matched sequences can be digested with restriction enzymes in order to release the cDNA inserts. If the plasmid does not contain the full length ortholog, the digests can be purified, *e.g.*, run on an agarose gel and the bands corresponding to the inserts can be cut from the gel and purified (Qiagen Gel Extraction kit). Such purified inserts are likely to contain overlapping regions which can be combined as templates of a PCR reaction using primers which are preferably located outside of the FabK open reading frame. The PCR reaction can be performed by RACE PCR, or by using ELONGASE (and its standard amplification system) supplied by Gibco-BRL, Gaithersburg, MD, under the following standard conditions: 5 minutes at 94°C; followed by 25 cycles of : 30 seconds at 94°C, 30 seconds at 50°C, and 3.5 minutes at 72°C; followed by 10 minutes at 72°C. Amplification should yield the expected product which can be ligated into a vector and used to transform an *E. coli* derivative via TA cloning (Invitrogen) for example. A resulting full-length FabK, for example, can be placed into an expression vector and the expressed recombinant FabK can then be assayed for enoyl reductase activity.

Alternatively, plasmids containing matched ortholog fragments can be used to transform competent bacteria (*e.g.*, from Gibco BRL, Gaithersburg MD). Bacteria can be streaked, then grown up overnight. Plasmid preps can be performed (*e.g.*, Qiagen Corp, Santa Clarita CA) and the plasmids can be digested by simultaneous

restriction digestion. Products of the digest can be separated by size on an agarose gel, for example, and purified. The corresponding bands cut from these gels can be ligated to form a full-length *fabK* cDNA and used to transform competent bacteria (DHFalpha) and the resulting plasmid can be purified.

In yet another embodiment, software programs such as the GCG package which includes a motif defining program "FindPatterns" can be used to identify a particular motif common to a family of proteins. This motif can then be used to identify other members of the family from publicly available databases. Using the motifs defined by SEQ ID NO:45 and SEQ ID NO:57, the sequences that were identified are shown in Table 3, below.

Expression of Enoyl Reductases

The present invention provides for expressing the nucleic acids which encode the enoyl reductases and fragments thereof, derivatives or analogs, and/or a functionally active derivative, including a chimeric protein, thereof, that has been inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein a "promoter." Thus, the nucleic acid encoding a bacterial FabK of the present invention for example is operationally associated with a promoter in an expression vector of the invention (see Example, below). Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. An expression vector also preferably includes a replication origin. One particular use for such expression vectors is to express a FabK protein in large quantities that can be used for functional and structural studies of the purified protein. The necessary transcriptional and translational signals can be provided on a recombinant expression vector, or they may be supplied by the native gene encoding FabK and/or its flanking regions.

Potential chimeric partners for the enoyl reductases of the present invention include glutathione-S-transferase (GST) or green fluorescent protein which may be useful in monitoring the cellular localization of the enoyl reductases.

Potential host-vector systems include but are not limited to bacterial cell systems, infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

A recombinant enoyl reductase protein of the invention, or functional fragment, derivative, chimeric construct, or analog thereof, may be expressed chromosomally, after integration of the coding sequence by recombination. In this regard, any of a number of amplification systems may be used to achieve high levels of stable gene expression [*See* Sambrook *et al.*, 1989, *supra*]. The cell containing the recombinant vector comprising the nucleic acid encoding the enoyl reductase is cultured in an appropriate cell culture medium under conditions that provide for expression of enoyl reductase by the cell.

Any of the methods previously described, or described in the Example below, for the insertion of DNA fragments into a cloning vector may be used to construct expression vectors containing a gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

Expression of the enoyl reductase may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control enoyl reductase gene expression include, the SV40 early promoter region [Benoist and Chambon, *Nature* **290:**304-310 (1981)], the promoter contained in the 3′ long terminal repeat of Rous sarcoma virus [Yamamoto, *et al.*, *Cell* **22:**787-797 (1980)], the herpes thymidine kinase promoter [Wagner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78:**1441-1445 (1981)], the regulatory sequences of the metallothionein gene [Brinster *et al.*, *Nature* **296:**39-

42 (1982)]; prokaryotic expression vectors such as the β-lactamase promoter [Villa-Kamaroff, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **75:**3727-3731 (1978)], or the *tac* promoter [DeBoer, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80:**21-25 (1983)]; *see also* "Useful proteins from recombinant bacteria" in *Scientific American* **242:**74-94 (1980); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells [Swift *et al.*, *Cell* **38:**639-646 (1984); Ornitz *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **50:**399-409 (1986); MacDonald, *Hepatology* **7:**425-515 (1987)]; insulin gene control region which is active in pancreatic beta cells [Hanahan, *Nature* **315:**115-122 (1985)], immunoglobulin gene control region which is active in lymphoid cells [Grosschedl *et al.*, *Cell* **38:**647-658 (1984); Adames *et al.*, *Nature* **318:**533-538 (1985); Alexander *et al.*, *Mol. Cell. Biol.* **7:**1436-1444 (1987)], mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells [Leder *et al.*, *Cell* **45:**485-495 (1986)], albumin gene control region which is active in liver [Pinkert *et al.*, *Genes and Devel.* **1:**268-276 (1987)], alpha-fetoprotein gene control region which is active in liver [Krumlauf *et al.*, *Mol. Cell. Biol.* **5:**1639-1648 (1985); Hammer *et al.*, *Science* **235:**53-58 (1987)], alpha 1-antitrypsin gene control region which is active in the liver [Kelsey *et al.*, *Genes and Devel.* **1:**161-171 (1987)], beta-globin gene control region which is active in myeloid cells [Mogram *et al.*, *Nature* **315:**338-340 (1985); Kollias *et al.*, *Cell* **46:**89-94 (1986)], myelin basic protein gene control region which is active in oligodendrocyte cells in the brain [Readhead *et al.*, *Cell* **48:**703-712 (1987)], myosin light chain-2 gene control region which is active in skeletal muscle [Sani, *Nature* **314:**283-286 (1985)], and gonadotropic releasing hormone gene control region which is active in the hypothalamus [Mason *et al.*, *Science* **234:**1372-1378 (1986)].

Expression vectors containing a nucleic acid encoding an enoyl reductase of the invention, for example FabK, can be identified by any number of general approaches

including: (a) PCR amplification of the desired plasmid DNA or specific mRNA, (b) nucleic acid hybridization, (c) presence or absence of selection marker gene functions, and (d) expression of inserted sequences. In the first approach, the nucleic acids can be amplified by PCR to provide for detection of the amplified product. In the second approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted marker gene. In the third approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "selection marker" gene functions (*e.g.*, β-galactosidase activity, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. In another example, if the nucleic acid encoding FabK is inserted within the "selection marker" gene sequence of the vector, recombinants containing the *FabK* insert can be identified by the absence of the *selection marker* gene function. In the fourth approach, recombinant expression vectors can be identified by assaying for the activity, biochemical, or immunological characteristics of the gene product expressed by the recombinant, provided that the expressed protein assumes a functionally active conformation. For example, the catalytic activity of the FabK can be tested.

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids col El, pCR1, pBR322, pMal-C2, pET, pGEX [Smith *et al.*, *Gene* **67**:31-40 (1988)], pMB9 and their derivatives, plasmids such as RP4; phage DNAS, e.g., the numerous derivatives of phage λ, e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2μ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or bacterial cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

For example, in a baculovirus expression systems, both non-fusion transfer vectors, such as but not limited to pVL941 (*Bam*H1 cloning site; Summers), pVL1393 (*Bam*H1, *Sma*I, *Xba*I, *Eco*R1, *Not*I, *Xma*III, *Bgl*II, and *Pst*I cloning site; Invitrogen), pVL1392 (*Bgl*II, *Pst*I, *Not*I, *Xma*III, *Eco*RI, *Xba*I, *Sma*I, and *Bam*H1 cloning site; Summers and Invitrogen), and pBlue*Bac*III (*Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site, with blue/white recombinant screening possible; Invitrogen), and fusion transfer vectors, such as but not limited to pAc700 (*Bam*H1 and *Kpn*I cloning site, in which the *Bam*H1 recognition site begins with the initiation codon; Summers), pAc701 and pAc702 (same as pAc700, with different reading frames), pAc360 (*Bam*H1 cloning site 36 base pairs downstream of a polyhedrin initiation codon; Invitrogen(195)), and pBlueBacHisA, B, C (three different reading frames, with *Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site, an N-terminal peptide for ProBond purification, and blue/white recombinant screening of plaques; Invitrogen (220)) can be used.

Expression vectors contemplated for use in the invention include vectors with inducible promoters, such as the dihydrofolate reductase (DHFR) promoter, *e.g.*, any expression vector with a *DHFR* expression vector, or a *DHFR*/methotrexate co-amplification vector, such as pED (*Pst*I, *Sal*I, *Sba*I, *Sma*I, and *Eco*RI cloning site, with the vector expressing both the cloned gene and *DHFR*; [*see* Kaufman, *Current Protocols in Molecular Biology*, **16**.12 (1991)]. Alternatively, a glutamine synthetase/methionine sulfoximine co-amplification vector, such as pEE14 (*Hind*III, *Xba*I, *Sma*I, *Sba*I, *Eco*RI, and *Bcl*I cloning site, in which the vector expresses glutamine synthase and the cloned gene; Celltech). In another embodiment, a vector that directs episomal expression under control of Epstein Barr Virus (EBV) can be used, such as pREP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive RSV-LTR promoter, hygromycin selectable marker; Invitrogen), pCEP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive hCMV immediate early gene, hygromycin selectable marker; Invitrogen), pMEP4 (*Kpn*I, *Pvu*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, *Bam*H1 cloning site, inducible methallothionein IIa gene promoter, hygromycin selectable marker: Invitrogen), pREP8 (*Bam*H1, *Xho*I, *Not*I, *Hind*III, *Nhe*I, and *Kpn*I cloning

site, RSV-LTR promoter, histidinol selectable marker; Invitrogen), pREP9 (*Kpn*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, and BamHI cloning site, RSV-LTR promoter, G418 selectable marker; Invitrogen), and pEBVHis (RSV-LTR promoter, hygromycin selectable marker, N-terminal peptide purifiable via ProBond resin and cleaved by enterokinase; Invitrogen). Selectable expression vectors for use in the invention include pRc/CMV (*Hind*III, *Bst*XI, *Not*I, *Sba*I, and *Apa*I cloning site, G418 selection; Invitrogen), pRc/RSV (*Hind*III, *Spe*I, *Bst*XI, *Not*I, *Xba*I cloning site, G418 selection; Invitrogen), and others. Vaccinia virus expression vectors (*see*, Kaufman, 1991, *supra*) for use according to the invention include but are not limited to pSC11 (*Sma*I cloning site, TK- and β-gal selection), pMJ601 (*Sal*I, *Sma*I, *Afl*I, *Nar*I, *Bsp*MII, *Bam*HI, *Apa*I, *Nhe*I, *Sac*II, *Kpn*I, and *Hind*III cloning site; TK- and β-gal selection), and pTKgptF1S (*Eco*RI, *Pst*I, *Sal*I, *Acc*I, *Hind*II, *Sba*I, *Bam*HI, and Hpa cloning site, TK or XPRT selection).

Yeast expression systems can also be used according to the invention to express the FabK protein. For example, the non-fusion pYES2 vector (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Gst*XI, *Eco*RI, *Bst*XI, *Bam*H1, *Sac*I, *Kpn*1, and *Hind*III cloning sit; Invitrogen) or the fusion pYESHisA, B, C (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Bst*XI, *Eco*RI, *Bam*H1, *Sac*I, *Kpn*I, and *Hind*III cloning site, N-terminal peptide purified with ProBond resin and cleaved with enterokinase; Invitrogen), to mention just two, can be employed according to the invention.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the

inserted sequences, or modifies and processes the gene product in the specific fashion desired. Different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification [*e.g.*, glycosylation, cleavage, (*e.g.*, of signal sequence) of proteins]. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an nonglycosylated core protein product. Expression in yeast can produce a glycosylated product. Furthermore, different vector/host expression systems may affect processing reactions, such as proteolytic cleavages, to a different extent.

Vectors are introduced into the desired host cells by methods known in the art, *e.g.*, transfection, transduction, electroporation, microinjection, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter [see, *e.g.*, Wu *et al.*, *J. Biol. Chem.* **267**:963-9670 (1992), Wu and Wu, *J. Biol. Chem.* **263**:14621-14624 (1988), Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990].

The present invention also provides cell lines made from cells transfected or transduced with the FabKs of the present invention. In one particular embodiment the cells are bacterial cells.

General Protein Purification Procedures:

The purification of FabK using a fusion protein that greatly simplifies the process is exemplified below. However, the present invention also provides a more general classical protein purification protocol. This procedure includes an initial step for purifying the enoyl reductases of the present invention, fragments thereof and related tagged or fusion proteins consisting of lysing the cells containing the enoyl reductases. Cell lysis can be achieved by a number of methods including through the use of a physical means such as a French press, a sonicator, or a blender; or through chemical means including enzymatic extractions (with for example, lysozyme or pancreatin), and/or organic extractions or solubilizations with detergents, such as

sodium dodecyl sulfate (SDS), Triton X-100, nonidet P-40 (NP-40), digoxin, sodium deoxycholate, and the like, including mixtures thereof; or through a combination of chemical and physical means. For example, solubilization can be enhanced by sonication of the suspension. Subsequent steps of purification include salting in or salting out, such as in ammonium sulfate fractionations; solvent exclusion fractionations, *e.g.*, an ethanol precipitation; detergent extractions to free the membrane bound enoyl reductases (if any) of the present invention using such detergents as Triton X-100, Tween-20 etc.; or high salt extractions. Solubilization of proteins may also be achieved using aprotic solvents such as dimethyl sulfoxide and hexamethylphosphoramide. In addition, high speed ultracentrifugation may be used either alone or in conjunction with other extraction techniques.

Generally good secondary isolation or purification steps include solid phase absorption using calcium phosphate gel or hydroxyapatite; or solid phase binding. Solid phase binding may be performed through ionic bonding, with either an anion exchanger, such as diethylaminoethyl (DEAE), or diethyl [2-hydroxypropyl] aminoethyl (QAE) SEPHADEX or cellulose; or with a cation exchanger such as carboxymethyl (CM) or sulfopropyl (SP) SEPHADEX or cellulose. Alternative means of solid phase binding includes the exploitation of hydrophobic interactions *e.g.*, the using of a solid support such as PHENYLSEPHAROSE and a high salt buffer; affinity-binding, using, *e.g.*, placing a substrate or substrate analog on to an activated support; immuno-binding, using *e.g.*, an antibody to a FabK of the present invention bound to an activated support; as well as other solid phase supports including those that contain specific dyes or lectins etc. A further solid phase support technique that is often used at the end of the purification procedure relies on size exclusion, such as SEPHADEX and SEPHAROSE gels, or pressurized or centrifugal membrane techniques, using size exclusion membrane filters.

Solid phase support separations are generally performed batch-wise with low-speed centrifugations or by column chromatography. High performance liquid chromatography (HPLC), including such related techniques as FPLC, is presently the most common means of performing liquid chromatography. Size exclusion

techniques may also be accomplished with the aid of low speed centrifugation.

In addition size permeation techniques such as gel electrophoretic techniques may be employed. These techniques are generally performed in tubes, slabs or by capillary electrophoresis.

Almost all steps involving protein purification employ a buffered solution. Unless otherwise specified, generally 25-100 mM concentrations are used. Low concentration buffers generally infer 5-25 mM concentrations. High concentration buffers generally infer concentrations of the buffering agent of between 0.1-2M concentrations. Typical buffers can be purchased from most biochemical catalogues and include the classical buffers such as Tris, pyrophosphate, monophosphate and diphosphate and the Good buffers [Good, *et al.*, *Biochemistry*, **5**:467 (1966); Good *et al. Meth. Enzymol.*, **24**: Part B, 53 (1972) ; and Fergunson, *et. al Anal. Biochem.* 104:300,(1980)] such as Mes, Hepes, Mops, tricine and Ches.

Materials to perform all of these techniques are available from a variety of sources such as Sigma Chemical Company in St. Louis, Missouri.

Antibodies to Enoyl Reductases

According to the present invention, an enoyl reductase such as a bacterial FabK of FabL protein obtained from a natural source or produced recombinantly or by chemical synthesis, and fragments or other derivatives or analogs thereof, including fusion proteins, may be used as an immunogen to generate antibodies that recognize the bacterial FabK or FabL polypeptide. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. The anti-FabK and anti-FabL antibodies of the invention may be cross reactive, *e.g.*, they may recognize a FabK or FabL from different species. Polyclonal antibodies have greater likelihood of cross reactivity. Alternatively, an antibody of the invention may be specific for a single ortholog of the enoyl reductases, such as *S. pneumoniae* FabK.

Various procedures known in the art may be used for the production of polyclonal antibodies to an enoyl reductase of the present invention or derivative or analog thereof. For the production of antibody, various host animals can be immunized by injection with a FabK, for example, or a derivative (*e.g.*, fragment or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, a FabK or fragment thereof can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed toward a FabK of the present invention, or fragment, analog, or derivative thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein [*Nature* **256**:495-497 (1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor *et al.*, *Immunology Today* **4**:72 1983); Cote *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**:2026-2030 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)]. In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology [PCT/US90/02545]. In fact, according to the invention, techniques developed for the production of "chimeric antibodies" [Morrison *et al.*, *J. Bacteriol.* **159**:870 (1984); Neuberger *et al.*, *Nature* **312**:604-608 (1984); Takeda *et al.*, *Nature* **314**:452-454 (1985)] by splicing the genes from a rabbit antibody molecule specific for a bacterial FabK, for example, together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or

disorders (described *infra*), since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves.

According to the invention, techniques described for the production of single chain antibodies [U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778] can be adapted to produce FabK-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse *et al.*, *Science* **246**:1275-1281 (1989)] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a FabK or its derivatives, or analogs.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab′ fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the

present invention. For example, to select antibodies which recognize a specific epitope of a FabK, for example the catalytic site, one may assay generated hybridomas for a product which binds to a FabK fragment containing such an epitope. For selection of an antibody specific to a FabK protein from a particular bacterium, one can select on the basis of positive binding with a bacterial FabK expressed by or isolated from cells of that bacterial species.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the enoyl reductase, such as FabK, for example using Western blotting, imaging FabK *in situ*, measuring levels thereof in appropriate physiological samples, etc. using any of the detection techniques mentioned above or known in the art. More particularly, the antibodies of the present invention can be used in flow cytometry studies, in immunohistochemical staining, and in immunoprecipitation which serves to aid the determination of the level of expression of a FabK protein.

In a specific embodiment, antibodies that agonize or antagonize the activity of a bacterial FabK can be generated. Such antibodies can be tested using the assays described herein.

Vaccination and Passive Immune Therapy

Active immunity against bacteria that rely on FabK and/or FabL for fatty acid synthesis can be induced by immunization (vaccination) with an immunogenic amount of FabK or FabL, or an antigenic derivative or fragment thereof, and an adjuvant, wherein the FabK and/or FabL, or antigenic derivative or fragment thereof, is the antigenic component of the vaccine. The protein, or fragment thereof can be conjugated to the carbohydrate capsule or capsules of one or more species of the bacterium. Covalent conjugation of a protein to a carbohydrate is well known in the art. Generally, the conjugation can proceed via a carbodiimide condensation reaction.

The FabK or FabL alone or conjugated to a capsule or capsules cannot cause bacterial infection, and the active immunity elicited by vaccination with the protein according

to the present invention can result in both an immediate immune response and in immunological memory, and thus provide long-term protection against infection by the bacterium. The FabK or FabL of the present invention, or antigenic fragments thereof, can be prepared in an admixture with an adjuvant to prepare a vaccine.

Selection of an adjuvant depends on the subject to be vaccinated. Preferably, a pharmaceutically acceptable adjuvant is used. For example, a vaccine for a human should avoid oil or hydrocarbon emulsion adjuvants, including complete and incomplete Freund's adjuvant. One example of an adjuvant suitable for use with humans is alum (alumina gel). A vaccine for an animal, however, may contain adjuvants not appropriate for use with humans.

An alternative to a traditional vaccine comprising an antigen and an adjuvant involves the direct *in vivo* introduction of DNA encoding the antigen into tissues of a subject for expression of the antigen by the cells of the subject's tissue. Such vaccines are termed herein "nucleic acid-based vaccines." For example, a naked DNA vector [*see, e.g.*, Ulmer *et al.*, *Science* **259**:1745-1749 (1993), a DNA vector transporter *e.g.*, Wu *et al.*, *J. Biol. Chem.* **267**:963-967 (1992); Wu and Wu, *J. Biol. Chem.* **263**:14621-14624 (1988); Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990], or a viral vector containing the desired FabK gene can be injected into tissue. Suitable viral vectors include retroviruses that are packaged in cells with amphotropic host range [*see* Miller, *Human Gene Ther.* **1**:5-14 (1990); Ausubel *et al.*, *Current Protocols in Molecular Biology*, § 9], and attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV) [*see, e.g.*, Kaplitt *et al.*, *Molec. Cell. Neurosci.* **2**:320-330(1991)], papillomavirus, Epstein Barr virus (EBV), adenovirus [*see, e.g.*, Stratford-Perricaudet *et al.*, *J. Clin. Invest.* **90**:626-630 (1992)], adeno-associated virus (AAV) [*see, e.g.*, Samulski *et al.*, *J. Virol.* **61**:3096-3101 (1987); Samulski *et al.*, *J. Virol.* **63**:3822-3828 (1989)], and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell.

Vectors containing the nucleic acid-based vaccine of the invention can be introduced

into the desired host by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, and/or a DNA vector transporter [*see* above, and U.S. Patent No. 5,916,879, Issued June 29, 1999, hereby incorporated by reference in its entirety].

Vaccines of the invention, can be administered by scarification, or via any parenteral route, including but not limited to intramuscular, intraperitoneal, intravenous, and the like. Preferably, since the desired result of vaccination is to elucidate an immune response to the antigen, and thereby to the pathogenic organism, administration directly, or by targeting or choice of a viral vector, indirectly, to lymphoid tissues, *e.g.*, lymph nodes or spleen. Since immune cells are continually replicating, they are ideal target for retroviral vector-based nucleic acid vaccines, since retroviruses require replicating cells.

Passive immunity can be conferred to an animal subject suspected of having a bacterial infection, for example, by administering antiserum, polyclonal antibodies, or a neutralizing monoclonal antibody against a Gram positive bacterium, for example, to the patient. Although passive immunity does not confer long term protection, it can be a valuable tool for the treatment of a bacterial infection of a subject who has not been vaccinated. Passive immunity is particularly important for the treatment of antibiotic resistant strains of Gram positive bacteria, for example, since no other therapy is available. Preferably, the antibodies administered for passive immune therapy are autologous antibodies. For example, if the subject is a human, preferably the antibodies are of human origin or have been "humanized," in order to minimize the possibility of an immune response against the antibodies.

The active or passive vaccines of the invention can be used alone or together as part of a multi-vaccine regimen to protect an animal subject from infection of a Gram positive bacteria, for example. Thus, a vaccine of the invention can be used in birds, such as chickens, turkeys, and pets; in mammals, preferably a human, although the vaccines of the invention are contemplated for use in other mammalian species,

including but not limited to domesticated animals (canine and feline); farm animals (bovine, ovine, equine, caprine, porcine, and the like); rodents; and undomesticated animals.

Assays for Identifying Agonists and Antagonists of FabK

Identification of the FabK protein and the FabL protein provides a basis for screening for drugs capable of specific interaction with the functionally relevant aspects of the protein. For example, an agonist or antagonist can be identified that stimulates or inhibits the FabK or FabL proteins. Since FabK and FabL play an important role in fatty acid synthesis such agonists and antagonists can be tested in a fatty acid synthetic assay as described in the Example below. Accordingly, in addition to rational design of compounds that bind to bacterial FabK or FabL, the present invention contemplates an alternative method for identifying specific agents that bind to FabK or FabL using the various screening assays known in the art.

Thus any screening technique known in the art can be used to screen for agonists or antagonists to the bacterial FabK or FabL proteins. The present invention contemplates screens for small molecule ligands or ligand analogs and mimics, as well as screens for natural ligands that bind to and antagonize FabK or FabL *in vivo*.

Knowledge of the primary sequence of a bacterial FabK protein of the present invention for example, and the similarity of that sequence with proteins of known function, can provide an initial clue as the agonists or antagonists of the protein. Identification and screening of antagonists is further facilitated by determining structural features of the bacterial FabK protein, *e.g.*, using X-ray crystallography, neutron diffraction, nuclear magnetic resonance spectrometry, and other techniques for structure determination. These techniques provide for the rational design or identification of agonists and antagonists.

Another approach uses recombinant bacteriophage to produce large libraries. Using the "phage method" [Scott and Smith, 1990, *Science* **249**:386-390 (1990); Cwirla, *et al.*, *Proc. Natl. Acad. Sci.*, **87**:6378-6382 (1990); Devlin *et al.*, *Science*, **249**:404-406

(1990)], very large libraries can be constructed (10^6-10^8 chemical entities). A second approach uses primarily chemical methods, of which the Geysen method [Geysen *et al.*, *Molecular Immunology* **23**:709-715 (1986); Geysen *et al. J. Immunologic Method* **102**:259-274 (1987)] and the method of Fodor *et al.* [*Science* **251**:767-773 (1991)] are examples. Furka *et al.* [*14th International Congress of Biochemistry, Volume 5*, Abstract FR:013 (1988); Furka, *Int. J. Peptide Protein Res.* **37**:487-493 (1991)], Houghton [U.S. Patent No. 4,631,211, issued December 1986] and Rutter *et al.* [U.S. Patent No. 5,010,175, issued April 23, 1991] describe methods to produce a mixture of peptides that can be tested as agonists or antagonists.

In another aspect, synthetic libraries [Needels *et al.*, *Proc. Natl. Acad. Sci. USA* **90**:10700-4 (1993); Ohlmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* **90**:10922-10926 (1993); Lam *et al.*, International Patent Publication No. WO 92/00252; Kocis *et al.*, International Patent Publication No. WO 9428028, each of which is incorporated herein by reference in its entirety], and the like can be used to screen for binding partners of the enoyl reductase, such as the bacterial FabK protein, that can potentially act as an antagonist of the protein.

The screening can be performed directly using peptides such as those corresponding to the catalytic domain of the bacterial FabK or FabL, or to any fragment and preferably active fragment of the FabK or FabL. Alternatively, chimeric proteins, which contain a fragment of the bacterial FabK or FabL may be used.

Screening can be performed with recombinant cells that express the bacterial FabK or FabL protein, or alternatively, using purified protein, and/or specific structural/functional domains of the bacterial FabK or FabL protein *e.g.*, produced recombinantly, as described above. For example, a labeled bacterial FabK protein can be used to screen libraries, as described in the foregoing references for small molecules that will inhibit the enoyl-ACP binding activity of the bacterial FabK protein.

The effective peptide(s) can be synthesized in large quantities for use in *in vivo*

models and eventually in humans to inhibit the FabK and/or FabL protein and thereby act as a drug that counteracts bacterial infection. It should be emphasized that synthetic peptide production is relatively non-labor intensive, easily manufactured, quality controlled and thus, large quantities of the desired product can be produced quite cheaply. Similar combinations of mass produced synthetic peptides have been used with great success [Patarroyo, Vaccine **10:**175-178 (1990)].

There are many additional methods for screening FabK or FabL inhibitors. For example, an enoyl reductase such as an enoyl-ACP reductase could be used with either enoyl-ACP as the substrate or preferably, using a substrate analog such as *trans*-2-enoyl-N-acetylcysteamine or an analogous enoyl-CoA. The reaction can be followed by oxidation of the reducing cofactor spectrophotometrically or fluorometrically [Heath *et al.*, *J. Biol. Chem.*, **273**:30316 (1998); Heath *et al.*, *J. Biol. Chem.*, **274**:11110-11114 (1999)] or using gel electrophoresis to separate labeled acyl-ACP products [Heath and Rock, *J. Biol. Chem.*, **271**:1833 (1996)]. Assays can also be designed to show FabK-cofactor-drug ternary complexes for example, using radiolabeled cofactor enzyme and drug [Heath *et al.*, *J. Biol. Chem.*, **274**:11110-11114 (1999)]. Since *S. pneumonia* FabK binds both NADH and FAD, either cofactor would be applicable. Additionally, FAD analogs including natural analogs such as FMN, or NADH analogs including natural analogs such as NADPH can be employed in certain assays. FabK activity can also be measured by cofactor oxidation in the absence of substrate. Alternatively, since the reaction is reversible, assays monitoring the reverse enzymatic reaction can be performed.

In addition, a scintillation proximity assay, filter precipitation assay, size exclusion assay or other methods that are based on the separation of the protein from a labeled cofactor and/or labeled substrate/product would also provide a reliable method for determining the catalytic activity of the FabK or FabL. Inhibitors would be identified when a significant decrease in the catalytic activity is determined.

As discussed below, the present invention also includes the use of the enoyl reductases and fragments that can be crystallized (*e.g.*, by X-ray crystallography) or

are soluble at relatively high concentrations (*e.g.*, for NMR analysis) for rational drug design. Potential effective drugs could be designed by molecular modeling of the FabK or FabL active site and then chemically synthesized or identified in existing drug libraries. Such drugs could be used to inactivate FabK or FabL and therefore, act as an anti-bacterial.

Labels

The reagents that contain the bacterial FabK or FabL proteins, or FabK or FabL fragments can be labeled for use in the screening assays. In one embodiment, the bacterial FabK or FabL proteins, or FabK or FabL fragments may be directly labeled including as part of a fusion protein, *e.g.*, with green fluorescent protein. In another embodiment, a labeled secondary reagent may be used to detect binding of the compound to a solid phase support containing a binding molecule of interest. Binding may be detected by *in situ* formation of a chromophore by an enzyme label. Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase. Other labels for use in the invention include colored latex beads, magnetic beads, fluorescent labels (e.g., fluorescein isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, Lucifer Yellow, AMCA blue, free or chelated lanthanide series salts, especially Eu^{3+}, to name a few fluorophores), chemiluminescent molecules, radio-isotopes, or magnetic resonance imaging labels.

Suitable labels include enzymes and proteins such as green fluorescent protein, fluorophores (*e.g.*, fluorescene isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, free or chelated lanthanide series salts, especially Eu^{3+}, to name a few fluorophores), chromophores, radioisotopes, chelating agents, dyes, colloidal gold, latex particles, ligands (*e.g.*, biotin), and chemiluminescent agents. When a control marker is employed, the same or different labels may be used for the receptor and control marker.

In the instance where a radioactive label, such as the isotopes ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re are used, known currently available counting procedures may be utilized. In the instance where the label is an enzyme,

detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art.

Direct labels are one example of labels which can be used according to the present invention. A direct label has been defined as an entity, which in its natural state, is readily visible, either to the naked eye, or with the aid of an optical filter and/or applied stimulation, e.g. U.V. light to promote fluorescence. Among examples of colored labels, which can be used according to the present invention, include metallic sol particles, for example, gold sol particles such as those described by Leuvering (U.S. Patent 4,313,734); dye sole particles such as described by Gribnau *et al.* (U.S. Patent 4,373,932) and May *et al.* (WO 88/08534); dyed latex such as described by May, *supra*, Snyder (EP-A 0 280 559 and 0 281 327); or dyes encapsulated in liposomes as described by Campbell *et al.* (U.S. Patent 4,703,017). Other direct labels include a radionucleotide, a fluorescent moiety or a luminescent moiety. In addition to these direct labelling devices, indirect labels comprising enzymes can also be used according to the present invention. Various types of enzyme linked immunoassays are well known in the art, for example, alkaline phosphatase and horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, urease, these and others have been discussed in detail by Eva Engvall in Enzyme Immunoassay ELISA and EMIT in *Methods in Enzymology*, **70**. 419-439, 1980 and in U.S. Patent 4,857,453.

Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase.

Other labels for use in the invention include magnetic beads or magnetic resonance imaging labels.

In another embodiment, a phosphorylation site can be created on an antibody of the invention for labeling with ^{32}P, *e.g.*, as described in European Patent No. 0372707 (application No. 89311108.8) by Sidney Pestka, or U.S. Patent No. 5,459,240, issued

Proteins, including the FabKs and FabLs of the present invention and antibodies thereto, can be labeled by metabolic labeling. Metabolic labeling occurs during *in vitro* incubation of the cells that express the protein in the presence of culture medium supplemented with a metabolic label, such as [^{35}S]-methionine (as described below in the Example) or [^{32}P]-orthophosphate. In addition to metabolic (or biosynthetic) labeling with [^{35}S]-methionine, the invention further contemplates labeling with [^{14}C]-amino acids and [^{3}H]-amino acids (with the tritium substituted at non-labile positions).

Solid Supports

A solid phase support for use in the present invention will be inert to the reaction conditions for binding. A solid phase support for use in the present invention must have reactive groups in order to attach a binding partner, such as an oligonucleotide encoding a bacterial FabK or FabL, a bacterial FabK or FabL fragment, or an antibody to a bacterial FabK or FabL, or for attaching a linker or handle which can serve as the initial binding point for any of the foregoing. In another embodiment, the solid phase support may be a useful chromatographic support, such as the carbohydrate polymers SEPHAROSE, SEPHADEX, agarose and agarose beads (as described in the Example below). As used herein, a solid phase support is not limited to a specific type of support. Rather a large number of supports are available and are known to any person having skill in the art. Solid phase supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, alumina gels, magnetic beads, membranes (including but not limited to nitrocellulose, cellulose, nylon, and glass wool), plastic and glass dishes or wells, etc. For example, solid phase supports used for peptide or oligonucleotide synthesis can be used, such as polystyrene resin (*e.g.*, PAM-resin obtained from Bachem Inc., Peninsula Laboratories, etc.), POLYHIPE® resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TentaGel®, Rapp Polymere, Tubingen, Germany) or polydimethylacrylamide resin (obtained from Milligen/Biosearch, California). In

synthesis of oligonucleotides, a silica based solid phase support may be preferred. Silica based solid phase supports are commercially available (*e.g.*, from Peninsula Laboratories, Inc.; and Applied Biosystems, Inc.).

Peptide Synthesis

Synthetic polypeptides, prepared using the well known techniques of solid phase, liquid phase, or peptide condensation techniques, or any combination thereof, can include natural and unnatural amino acids. Amino acids used for peptide synthesis may be standard Boc (N^{α}-amino protected N^{α}-t-butyloxycarbonyl) amino acid resin with the standard deprotecting, neutralization, coupling and wash protocols of the original solid phase procedure of Merrifield [*J. Am. Chem. Soc.*, **85:**2149-2154 (1963)], or the base-labile N^{α}-amino protected 9-fluorenylmethoxycarbonyl (Fmoc) amino acids first described by Carpino and Han [*J. Org. Chem.*, **37:**3403-3409 (1972)]. Both Fmoc and Boc N^{α}-amino protected amino acids can be obtained from Fluka, Bachem, Advanced Chemtech, Sigma, Cambridge Research Biochemical, Bachem, or Peninsula Labs or other chemical companies familiar to those who practice this art. In addition, the method of the invention can be used with other N^{α}-protecting groups that are familiar to those skilled in this art. Solid phase peptide synthesis may be accomplished by techniques familiar to those in the art and provided, for example, in Stewart and Young, 1984, Solid Phase Synthesis, Second Edition, Pierce Chemical Co., Rockford, IL; Fields and Noble, 1990, Int. J. Pept. Protein Res. 35:161-214, or using automated synthesizers, such as sold by ABS. Thus, enoyl reductases of the invention may comprise D-amino acids, a combination of D- and L-amino acids, and various "designer" amino acids (*e.g.*, β-methyl amino acids, Cα-methyl amino acids, and Nα-methyl amino acids, etc.) to convey special properties. Synthetic amino acids include ornithine for lysine, fluorophenylalanine for phenylalanine, and norleucine for leucine or isoleucine. Additionally, by assigning specific amino acids at specific coupling steps, α-helices, β turns, β sheets, γ-turns, and cyclic peptides can be generated.

Rational Drug Design

The present invention provides a FabK and a FabL that can be crystallized or alternatively modified (such as proteolytically cleaved to its catalytic core) and then crystallized into a crystal that effectively diffracts X-rays for the determination of the atomic coordinates of the FabK or FabL to a resolution of better than 5.0 Angstroms and preferably to a resolution equal to or better than 3.5 Angstroms. The FabK or FabL can be expressed either as described below in the Example, or as described above. Of course, the FabKs or FabLs provided herein serve only as example, since crystallization can tolerate a broad range of active FabKs and FabLs. Therefore, any person with skill in the art of protein crystallization having the present teachings and without undue experimentation could crystallize a large number of forms of the FabK and FabL from a variety of core FabK or FabL fragments for example, or alternatively using a full length FabK or FabL from a related source. As mentioned above, a FabK or FabL having conservative substitutions in its amino acid sequence is also included in the invention, including a selenomethionine substituted form.

Crystals of the FabK or FabL of the present invention can be grown by a number of techniques including batch crystallization, vapor diffusion (either by sitting drop or hanging drop) and by microdialysis. Seeding of the crystals in some instances is required to obtain X-ray quality crystals. Standard micro and/or macro seeding of crystals may therefore be used.

Crystals can be characterized by using X-rays produced in a conventional source (such as a sealed tube or a rotating anode) or using a synchrotron source. Methods of characterization include, but are not limited to, precision photography, oscillation photography and diffractometer data collection. Selenium-Methionine may be used, or alternatively a mercury derivative data set (*e.g.*, using PCMB) could be used in place of the Selenium-Methionine derivatization. Cells can be induced to incorporate selenomethionine by suppression of methionine biosynthesis [Doublie, *Methods Enzymol.*, **276**:523-530 (1997)].

Structural determinations can be performed by calculating Patterson maps using

PHASES [Furey and Swaminathan, *Methods Enzymol.*, **277**:590-620 (1997)] for the ethyl-$HgCl_2$ and Ta_6Br_{14} derivatives and using the Pb-derivative as native for example. The location of a particular site, such as a flavin binding site of the FabK, for example or the entire catalytic site can be derived manually for example, and then confirmed using HEAVY [Terwilliger *et al.*, *Acta Cryst.*, **A 43**:34-38 (1987)] for each derivative, and cross-confirmed using difference Fourier techniques. Additional sites, as well as sites for heavy-metal derivatives, can be obtained using difference Fourier techniques. The final phasing calculations can be performed using SHARP [LaFortelle *et al.*, *Crystallographic Computing*, (Eds. Bourne and Watenpaugh) 1997)]. If large errors between groups of data from each synchrotron beamline is found, multiple sets from CHESS A1 may need to be initially refined with SHARP. Other groups of data can be subsequently included but with the refined heavy-atom parameters for the previously refined data sets fixed for all subsequent refinements. After each trial refinement, density modification and phase extension can be performed using SOLOMON.

Map interpretation and model building can be performed using O [Jones *et al.*, *Acta Cryst*, **A 47**:110-119 (1991)]. Refinement calculations can be performed using CNS [Adams *et al.*, *Proc. Natl. Acad. Sci. USA*, **94**:5018-5023 (1997)].

Once the three-dimensional structure of a crystal comprising a FabK or FabL is determined, (or determined by an alternative methodology such as NMR) a potential modulator of FabK or FabL can be examined through the use of computer modeling using a docking program such as GRAM, DOCK, or AUTODOCK [Dunbrack *et al.*, *Folding & Design*, **2**:27-42 (1997)]. This procedure can include computer fitting of potential modulators to the FabK or FabL to ascertain how well the shape and the chemical structure of the potential modulator will bind to FabK or FabL [Bugg *et al.*, *Scientific American*, **Dec.**:92-98 (1993); West *et al.*, *TIPS*, 16:67-74 (1995)]. Computer programs can also be employed to estimate the attraction, repulsion, and steric hindrance of the FabK or FabL with a modulator/inhibitor (*e.g.*, the FabK or FabL and a potential stabilizer).

Generally the tighter the fit, the lower the steric hindrances, and the greater the attractive forces, the more potent the potential modulator since these properties are consistent with a tighter binding constant. Furthermore, the more specificity in the design of a potential drug the more likely that the drug will not interact as well with other proteins. This will minimize potential side-effects due to unwanted interactions with other proteins.

Initially compounds found to bind bacterial FabK or FabL, by high throughput screening for example, can be systematically modified by computer modeling programs until one or more promising potential analogs are identified. In addition, selected analogs can then be systematically modified by computer modeling programs until one or more potential analogs are identified. Such analysis has been shown to be effective in the development of HIV protease inhibitors [Lâm *et al.*, *Science* **263**:380-384 (1994); Wlodawer *et al.*, *Ann. Rev. Biochem.* **62**:543-585 (1993); Appelt, *Perspectives in Drug Discovery and Design* **1**:23-48 (1993); Erickson, *Perspectives in Drug Discovery and Design* **1**:109-128 (1993)]. Thus a potential modulator could be obtained by initially screening a random peptide library produced by recombinant bacteriophage for example, [Scott and Smith, *Science,* **249:**386-390 (1990); Cwirla *et al., Proc. Natl. Acad. Sci.,* **87:**6378-6382 (1990); Devlin *et al.,* *Science,* **249:**404-406 (1990)]. A peptide selected in this manner would then be systematically modified by computer modeling programs as described above, and then treated analogously to a structural analog as described above.

Once a potential modulator/inhibitor is identified it can be either selected from a library of chemicals as are commercially available from most large chemical companies including Merck, GlaxoWelcome, Bristol Meyers Squib, Monsanto/Searle, Eli Lilly, Novartis and Pharmacia UpJohn, or alternatively the potential modulator may be synthesized *de novo*. As mentioned above, the *de novo* synthesis of one or even a relatively small group of specific compounds is reasonable in the art of drug design. The potential modulator can be placed into a standard binding and/or catalytic assay with FabK or FabL, or an active fragment thereof, for example. The fragments can be synthesized by either standard peptide synthesis

described above, or generated through recombinant DNA technology or classical proteolysis. Alternatively the corresponding full-length proteins may be used in these assays.

For example, the FabK or a fragment thereof can be attached to a solid support. Methods for placing the FabK on the solid support are well known in the art and include such things as linking biotin to the FabK and linking avidin to the solid support. The solid support can be washed to remove unreacted species. A solution of a labeled potential modulator (*e.g.*, an inhibitor) can be contacted with the solid support. The solid support is washed again to remove the potential modulator not bound to the support. The amount of labeled potential modulator remaining with the solid support and thereby bound to the FabK can be determined. Alternatively, or in addition, the dissociation constant between the labeled potential modulator and the FabK, for example can be determined. Suitable labels for either the bacterial FabK subunit or the potential modulator are exemplified herein. In a particular embodiment, isothermal calorimetry can be used to determine the stability of the bacterial FabK in the absence and presence of the potential modulator.

In another embodiment, a Biacore machine can be used to determine the binding constant of the bacterial FabK or FabL to cofactors, substrates, products or analogs thereof in the presence and absence of the potential modulator. Alternatively, the bacterial FabK or FabL can be immobilized on a sensor chip.

In this case the dissociation constant for the bacterial FabK or FabL can be determined by monitoring changes in the refractive index with respect to time as buffer is passed over the chip. [O'Shannessy *et al.* Anal. Biochem. **212**:457-468 (1993); Schuster *et al.*, *Nature* **365**:343-347 (1993)]. Scatchard Plots, for example, can be used in the analysis of the response functions using different concentrations of a FabK for example. Flowing a potential modulator at various concentrations over the bacterial FabK and monitoring the response function (*e.g.*, the change in the refractive index with respect to time) allows the dissociation constant to be determined in the presence of the potential modulator and the bacterial FabK

cofactors and/or substrates or products or their analogs, and thereby indicates whether the potential modulator is either an inhibitor, or an agonist of the bacterial FabK.

In another aspect of the present invention a potential modulator is assayed for its ability to inhibit the bacterial FabK or FabL. A modulator that inhibits the FabK or FabL can then be selected. In a particular embodiment, the effect of a potential modulator on the catalytic activity of bacterial FabK or FabL is determined. The potential modulator is then be added to a bacterial culture to ascertain its effect on bacterial proliferation. A potential modulator that inhibits bacterial proliferation can then be selected.

In a particular embodiment, the effect of the potential modulator on the catalytic activity of the bacterial FabK or FabL is determined (either independently, or subsequent to a binding assay as exemplified above). In one such embodiment, the rate of the enoyl reductase is determined. For such assays the oxidation/reduction of a cofactor can be determined. This assay can be performed using a real-time assay *e.g.*, with a spectrophotometer. Alternatively, the determination can include the withdrawal of aliquots from the incubation mixture at defined intervals and subsequent placing of the aliquots on nitrocellulose paper or on gels. In a particular embodiment the potential modulator is selected when it is an inhibitor of the bacterial FabK.

When suitable potential modulators are identified, a supplemental crystal can be grown which comprises the bacterial FabK or FabL and the potential modulator. Preferably the crystal effectively diffracts X-rays for the determination of the atomic coordinates of the protein-ligand complex to a resolution of better than 5.0 Angstroms, more preferably equal to or better than 3.5 Angstroms. The three-dimensional structure of the supplemental crystal can be determined by Molecular Replacement Analysis. Molecular replacement involves using a known three-dimensional structure as a search model to determine the structure of a closely related molecule or protein-ligand complex in a new crystal form. The measured X-ray diffraction properties of the new crystal are compared with the search model

structure to compute the position and orientation of the protein in the new crystal. Computer programs that can be used include: X-PLOR (see above), CNS, (Crystallography and NMR System, a next level of XPLOR), and AMORE [J. Navaza, *Acta Crystallographics ASO,* 157-163 (1994)]. Once the position and orientation are known an electron density map can be calculated using the search model to provide X-ray phases. Thereafter, the electron density is inspected for structural differences and the search model is modified to conform to the new structure. Using this approach, it will be possible to use a crystal of the bacterial FabK or FabL to solve the three-dimensional structures of other bacterial core FabKs or FabLs having pre-ascertained amino acid sequences. Other computer programs that can be used to solve the structures of the bacterial FabK from other organisms include: QUANTA, CHARMM; INSIGHT; SYBYL; MACROMODE; and ICM.

A candidate drug can be selected by performing rational drug design with the three-dimensional structure determined for the supplemental crystal, preferably in conjunction with computer modeling discussed above. The candidate drug (*e.g.*, a potential modulator of bacterial FabK) can then be assayed as exemplified above, or *in situ.* A candidate drug can be identified as a drug, for example, if it inhibits bacterial proliferation.

A potential inhibitor (*e.g.*, a candidate drug) would be expected to interfere with bacterial growth. Therefore, an assay that can measure bacterial growth may be used to identify a candidate drug.

Methods of testing a potential bactericidal agent (*e.g.*, the candidate drug) in an animal model are well known in the art, and can include standard bactericidal assays. The potential modulators can be administered by a variety of ways including topically, orally, subcutaneously, or intraperitoneally depending on the proposed use. Generally, at least two groups of animals are used in the assay, with at least one group being a control group which is administered the administration vehicle without the potential modulator.

For all of the drug screening assays described herein further refinements to the structure of the drug will generally be necessary and can be made by the successive iterations of any and/or all of the steps provided by the particular drug screening assay.

The present invention also includes the drugs that are obtained by the methods of the present invention.

The present invention may be better understood by reference to the following non-limiting Example, which is provided as exemplary of the invention. The following example is presented in order to more fully illustrate the preferred embodiments of the invention. It should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE

THE ENOYL-ACP REDUCTASE II OF *STREPTOCOCCUS PNEUMONIAE* (FABK) IS A FLAVOPROTEIN THAT CONFERS HIGH LEVEL TRICLOSAN RESISTANCE TO *ESCHERICHIA COLI*

Introduction

Fatty acid biosynthesis in bacteria is accomplished by a set of discrete proteins that each catalyze a specific step in the pathway and are encoded by individual genes [Rock and Cronan, *Biochim. Biophys. Acta,* **1302**:1 (1996)]. The NADH-dependent enoyl-ACP reductase (FabI) catalyzes the last reaction in each cycle of 2-carbon fatty acyl chain elongation. This enzyme plays a role in determining the rate of fatty acid production through feedback regulation by long-chain acyl-ACP end-products of the pathway [Heath and Rock, *J. Biol. Chem.*, **271**:1833 (1996)]. As disclosed below, an alternative enzyme has been isolated which carries out the identical catalytic reaction as FabI. The characteristics of this new enzyme are also disclosed.

Methods

Preparation of nucleic acids encoding FabK: The synthetic primers: 5'-TCTAGACATAT GAAAACGCGTATTACAGAATTA-3' (SEQ ID NO:59) and 5'-GGATCCTAGATACTGGGCACCTTGACC-3' (SEQ ID NO:60) were used to amplify a band of 1030 bp from the chromosome of *S. pneumoniae* strain R6 in a reaction containing 10 μM of each primer, 500 μM dNTPs and 4 mM $MgCl_2$ in 1 x Buffer B from Promega. The reaction was heated to 95°C for 5 minutes to effect lysis of the cells prior to the addition of Taq DNA polymerase to 1.25 U per reaction. 35 cycles of 95°C for 15 seconds; 55 °C for 15 seconds, and 72°C for 2 minutes were performed. The PCR product was purified following gel electrophoresis and cloned into the pCR2.1 vector (Invitrogen). The gene was then subcloned into the *Nde*I and *Bam*HI sites of the plasmid pET-15b (Novagen) to form pET-fabK for inducible expression of FabK with an amino-terminal 6xHis-tag. The gene was sequenced to verify that the expected sequence had been obtained. pET-fabK was digested with *Xba*I and *Bam*HI and the orf subcloned into pBluescript KSII (+) (Stratagene) digested with the same enzymes to create pfabK to constitutively express the His-tagged FabK. *E. coli* strain RJH13 (*fabI*(Ts)) was transformed with either pBluescript (empty vector), pfabI [Heath *et al.*, *J. Biol. Chem.*, **273**:30316 (1998)] or pfabK. Cells were grown at the permissive temperature for the host strain (30°C), and then individual colonies spotted to LB agar plates and incubated at 42°C. Plasmids pfabI and pfabK complemented the growth phenotype, while no growth was seen when cells were transformed with the empty vector.

Expression and purification of His-FabK in E. coli and purification of the protein. The plasmid pET-fabK was transformed into *E. coli* strain BL21-Codon Plus-(DE3)-R1L (Stratagene) and cells were grown at 37°C in liquid LB medium plus ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) to a density of approximately 5 x 10^8 cells per milliliter. IPTG was added to a 1 mM concentration and 0.5mg/ml of riboflavin was added. Growth was continued for 3 hours at 37°C, and the cells were harvested by centrifugation. The cells were then resuspended in 20 mM Tris, pH 7.9 containing 0.5 M NaCl, 1 mM PMSF and 10% glycerol (MCAC buffer) and lysed by adding 0.1 mg/ml lyzozyme and 1% Triton X-100. The extract was then frozen at

-70°C . After gently thawing the extract on ice, it was centrifuged at 50,000 rpm in a 70.1 Ti rotor for one hour. Soluble protein was then applied to a 6 ml (bed volume) Ni-NTA column (Qiagen) that had been previously equilibrated with MCAC buffer. The column was then washed with 5 column volumes of MCAC buffer and then 5 column volumes of MCAC buffer plus 40 mM imidazole. The His-tagged protein was eluted with MCAC buffer plus 200 mM imidazole. The purified protein migrated at 36 kDa on SDS gel electrophoresis corresponding to the predicted molecular weight plus the His-tag (predicted size 36,340) and was greater than 98% pure as judged by SDS-PAGE with Coomassie blue staining.

Enzymatic Assays: A reaction mixture containing 100 μM ACP, 1 mM β-mercaptoethanol, 100 μM acetyl-CoA, 50 μM [2-^{14}C]malonyl-CoA (56 mCi/mmol), 200 μM NADPH, 200 μM NADH, and 12.5 μg/ml of each FabD, FabH, FabG, FabA and FabZ in 0.1 M sodium phosphate pH 7.0 was incubated at 37°C for 30 min to generate the *trans*-2-enoyl-ACP substrate before being aliquoted into individual reaction tubes to which FabK was added to the final amount as indicated for the Figures, above. The final reaction volume was 40 μL. Reactions were then incubated for 30 min at 37 °C and were stopped by placing into an ice slurry. Gel loading buffer was added, and the entire sample was loaded onto a 13% polyacrylamide gel containing 0.5 M urea [Heath and Rock, *J. Biol. Chem.*, 271:1833 (1996)].

Results

Only a single type of enoyl-ACP reductase, typified by the *E. coli* FabI, is known [Bergler *et al.*, *J. Biol. Chem.*, **269**:5423 (1994), and Heath and Rock, *J. Biol. Chem.*, **270**:26538 (1995)]. As anticipated, FabI is widely distributed in both Gram negative and Gram positive bacteria because enoyl-ACP reduction is a required chemical step for this biosynthetic pathway of saturated fatty acids. However surprisingly, homologs to FabI protein are conspicuously absent from several bacterial genome databases at the TIGR website (http:\\www.tigr.org) (*see* below, and Table 1).

Table 1.

OCCURRENCE OF *FABI* AND *FABK* IN MICROORGANISMS

Organism	% Identity to[a]		
	FabI	FabK	FabL
Escherichia coli	100	—	—
Streptococcus pneumoniae	—	100	—
Aquifex aeolicus	49	—	—
Archeaglobus fugidis	—	37	—
Bacillus subtilis	51	—	100
Bordetella pertussis	65	—	—
Campylobacter jejuini	47	—	41
Chlamydia pneumoniae	34	—	—
Clostridium acetobutyricum	—	58	—
Haemophilus influenza	75	—	—
Helicobacter pylori	45	—	40
Mycobacterium tuberculosis	33	31	—
Neisseria gonorrhoeae	61	—	—

Rickettsia prowazekii	43	—	—
Thermatoga maritima	—	48	—
Enterococcus feacalis	47	68	—
Pseudomonas aeruginosa	69	33	—
Staphylococcus aureus	43	—	—
Streptomyces collinus	—	—	38

[a]The *E. coli* FabI, *S. pneumoniae* FabK, and *B. subtilus* FabL protein sequences were used to search the dynamically translated unfinished and complete microbial genomes database at NCBI for open reading frames encoding homologous proteins. Open reading frames were identified by using the tblastn algorithm. Criteria for identification were: a highly significant score (expected match = 10^{-30} or lower) from the initial search, the presence of an open reading frame encoding a predicted protein of approximately the same size as the characterized proteins, and a satisfactory alignment with FabI and FabK using the PileUp algorithm in the GCG package. The number of identical residues was then scored. — indicates that homologous genes were not detected in an organism with a completely sequenced genome.

FabI homologs are easily recognized in bacterial genomes using BLAST search algorithms (Table 1). However, database searches using the known *E. coli* enoyl-ACP reductase (FabI) protein sequence failed to reveal the presence of a gene encoding a homolog of this protein in any of the Streptococcal genomes including *S. pyogenes, S. mutans, and S. pneumoniae*, even though the complete genomes for these three Streptococcal strains are essentially fully sequenced. In direct contrast, homologs of all of the other proteins required in fatty acid synthesis (*i.e.*, FabD, FabH, FabG, FabF, Fab Z, ACP and the four subunits of ACC) were all readily recognizable by performing a similiar tblastn search of the identical databases with the corresponding amino acid sequences. Indeed, FabI homologs were also not found in *Clostridium acetobutyricum, C. difficile* and *Thermatoga maritima*. Since enoyl-ACP reduction is a required chemical step in the synthesis of an acyl chain by this pathway, these organisms must contain a novel gene that encodes an enoyl-ACP reductase with a distinctly different primary structure than FabI. This gap in the understanding of type II fatty acid synthases and the importance of enoyl-ACP reductase as a target for the development of new therapeutics prompted the investigation of the reductase step in the type II system of *S. pneumoniae*.

Importantly, all of the other proteins required in fatty acid synthesis were present on a single contig (Contig sp _ 90 in the January 28, 1999 release). Indeed, the analysis of the *fab* genes in all three Streptococci genomes revealed that the entire set of *fab* genes was present in a cluster spanning about 10 kbp (Fig. 1A). A similar *fab* gene cluster was found in *C. difficile*. Although clusters of *fab* genes, except *fabI*, are present in other bacteria such as *B. subtilis* and *E. coli*, these clusters contain only a subset of the required genes and the other *fab* genes are sprinkled throughout the genome [Rock and Cronan *Biochim. Biophys. Acta*, **1302**:1 (1996)].

Further analysis indicated that the genes were clustered together with an apparent open reading frame for an heretofore unreported protein. For example, the order of proteins was: fabH-acpP-unknown-fabD-fabF-fabG-bccP-fabZ-bccA-accD-accA. The unidentified open reading frame contained within the Streptococci and Clostridial clusters was named *fabK*. FabK is predicted to encode a protein of approximately 34

kDa containing a centrally located FAD binding domain as defined by Nagy *et al.*, [*Proc. Natl. Acad. Sci. U.S.A.*, **89**:8966 (1992)] (Fig. 1B). *S. pyogenes* and *S. mutans* had *fabK* located in the same position in the *fab* cluster with identity at the protein sequence level to *S. pneumoniae* of 78% and 73%, respectively. Alignment of representative FabK proteins from these organisms revealed highly related amino terminal and central regions and divergent carboxy terminal domains (Fig. 1B). The association of *fabK* with the Streptococci *fab* cluster led to the hypothesis that this gene encoded a unique enoyl-ACP reductase.

The unknown protein was used to search the GenBank non-redundant database. The search revealed that FabK was present in a variety of microorganisms including bacteria, Archae, yeast and fungi (Table 1). Indeed, the highest scoring match to a known protein was Expect=4e09 to the 2-nitropropane diooxygenase (SwissProt: 2NPD_WILMR) from yeast *Williopsis saturnus*. Higher scoring hypothetical proteins in several bacteria were noted, most of which had been annotated as nitropropane diooxygenases (*e.g.*, a protein from the archeabacteria *Archaeoglobus fulgidus* with E= 7e-46) based on a low level of sequence identity to the yeast protein. Thus, the unknown gene, and related genes in other bacteria, shared some sequence homology to the 2NPD of yeast, but this similarity was not sufficient to conclude that the proteins had the same function. Importantly, the nitropropane diooxygenase activity of 2NPD was believed to be an ancillary role for the enzyme in yeast, indicating that heretofore, the natural role of this enzyme had not be ascertained.

The identification of FabK as an enoyl-ACP reductase was established by biochemical characterization of the purified enzyme. The *fabK* gene was amplified from *S. pneumoniae* strain R6 chromosomal DNA and cloned into the pET-15b expression vector (see Methods, above). The yellow protein was purified to homogeneity and exhibited an apparent molecular weight equivalent to the predicted mass of FabK plus the His-tag (Fig. 2A). Spectral analysis of freshly purified FabK revealed the presence of 0.8 moles of FAD per mole of FabK monomer (Fig. 2B). The FAD cofactor was tightly, but not covalently bound, and the FAD content was progressively reduced during prolonged dialysis. The protein was tested for

enoyl-ACP reductase activity in an *in vitro* coupled assay system utilizing purified *E. coli* Fab proteins to generate the *trans*-2-butenoyl-ACP substrate (Fig. 2C). FabK had a specific activity of 66 ± 4 nmole product formed/min/mg FabK in this assay (Fig. 2D). NADH was an essential requirment for enoyl-ACP reductase and FabK carried out the slow oxidation of NADH, but not NADPH, in the absence of substrate. Triclosan (25 μg/ml) did not inhibit FabK activity in the *in vitro* assay. These data illustrate that FabK is a flavoprotein that possesses *trans*-2-enoyl-ACP reductase activity.

FabK was able to functionally replace the FabI enoyl-ACP reductase *in vivo*. It also confers triclosan resistance to *E. coli*. FabI is the only enoyl reductase in *E. coli* and strain RJH13 harbors a *fabI*(Ts) allele and fails to grow at 42°C [Heath *et al.*, *J. Biol. Chem.*, **273**:30316 (1998)]. Transformation of strain RJH13 with pfabK restored the ability of strain RJH13 to grow at the non-permissive temperature, thus illustrating that FabK substituted for all of the functions of FabI (see Methods, above). The pfabK plasmid was then introduced into the wild-type *E. coli* strain W3110. Strain W3110 was sensitive to triclosan and either the presence of the pFabI multi-copy plasmid or the chromosomal *fabI*[G93V] mutant increased triclosan resistance (Table 2). This result is understood based on the observation that while the FabI[G93V] mutant fails to form a high-affinity FabI-NAD^+-triclosan ternary complex, it is still inhibited by the drug [Heath *et al.*, *J. Biol. Chem.*, **274**:11110-11114 (1999)]. Introduction of the pfabK plasmid into strain W3110 shifted the minimum inhibitory concentration (MIC) for triclosan to greater than 2 mg/ml (Table 2). These cells are completely resistant to triclosan confirming that FabK is not a target for this drug and that FabI is the only target for triclosan in *E. coli*. *P. aeruginosa* is unique among Gram negative bacteria in that it contains both FabI and FabK in its genome (Table 1), and is completely refractory to triclosan inhibition [Bhargava and Leonard, *Am. J. Infect. Control*, **24**:209 (1996)]. Furthermore, the FabI protein from *P. aeruginosa* is highly susceptible to triclosan *in vitro* and disruption of the *fabI* gene in this organism does not result in an obvious growth phenotype [Hoang and Schweizer, *J. Bacteriol.*, **181**:5489 (1999)] suggesting the presence of a second enoyl reductase.

Table 2.

FabK expression confers triclosan resistance to makes *E. coli*.

Strain	MIC[a] (μg/ml)
E. coli W3110/pBluescript	0.25
E. coli RJH108 (*fabI*[G93V])[b]	16
E. coli W3110/pfabI[c]	2
E. coli W3110/pfabK[c]	>2,000
S. pneumoniae R6	2

[a]Minimum inhibitory concentrations for *E. coli* strains were tested by spotting at least six single colonies onto a series of LB agar plates containing different concentrations of triclosan. The *S. pneumoniae* MIC was determined by spreading 10 μL of culture just entering stationary phase onto brain heart infusion plates containing triclosan. The MIC reported in each case is the concentration of triclosan at which no growth was observed in at least three separate experiments. The *S. pneumoniae* MIC was similar to that previously published for Streptococci [Bhargava and Leonard, *Am. J. Infect. Control*, **24**:209 (1996)].

[b]Strain RJH108 was a spontaneously occurring triclosan resistant derivative of W3110 [Heath *et al.*, *J. Biol. Chem.*, **273**:30316 (1998)].

[c]pfabI expresses the His-tag FabI from *E. coli* [Heath *et al.*, *J. Biol. Chem.*, **273**:30316 (1998)] and pfabK expresses the His-tag FabK protein using the same vector and construction method as employed for pfabI.

Interestingly, FabK does not confer the same high level of triclosan resistance to *S. pneumoniae* (Table 2) and other Gram positive bacteria that contain *fabK* [Bhargava and Leonard, *Am. J. Infect. Control*, **24**:209 (1996)]. These data suggest that triclosan has a second target in Gram positive bacteria that is absent from Gram negative organisms. The triclosan MIC for *S. pneumoniae* and *B. subtilis* are both about 2 μg/ml which is about an order of magnitude higher than typical for Gram negative bacteria (Table 2) [Bhargava and Leonard, *Am. J. Infect. Control*, **24**:209 (1996)]. This suggests that the Gram positive FabI can be compensated for by FabK or FabL up to the concentration of triclosan required to inhibit the second, less sensitive triclosan target.

The yeasts *W. saturnus* and *Saccharomyces cerevisiae* (ORF YJR149w) also contain *fabK* homologs (Table 1). These FabKs are less closely related to *S. pneumoniae fabK* than the bacterial proteins (Table 1) and the eukaryotic enzymes contain inserts of approximately 30 and 8 amino acids that are also observed in the *B. subtilis* and *S. aureus* FabKs (Fig. 1). The *W. saturnus* enzyme has been purified and characterized as a NADH-dependent FAD-containing 2-nitropropane dioxygenase [Tchorzewski *et al.*, *Eur. J. Biochem.*, **226**:841 (1994)]. Nitropropane is an industrial environmental pollutant, the enzyme has a low turnover rate, and the nitropropane substrate forms a covalent adduct with the FAD cofactor that inactivates the enzyme [Heasley and Fitzpatrick, *Biochem. Biophys. Res. Commun.*, **225**:6 (1996); Gadda *et al.*, *J. Biol. Chem.*, **272**:5563 (1997); Gadda and Fitzpatrick, *Biochemistry*, **37**:6154 (1998); and Gadda and Fitzpatrick, *Arch. Biochem. Biophys.*, **363**:309 (1999)]. These observations argue that this enzyme performs another function in the cells [Tchorzewski *et al.*, *Eur. J. Biochem.*, **226**:841 (1994) and Heasley and Fitzpatrick, *Biochem. Biophys. Res. Commun.*, **225**:6 (1996); Gadda *et al.*, *J. Biol. Chem.*, **272**:5563 (1997); Gadda and Fitzpatrick, *Biochemistry*, **37**:6154 (1998); and Gadda and Fitzpatrick, *Arch. Biochem. Biophys.*, **363**:309 (1999)].

The significance of the FAD cofactor in FabK is not immediately obvious. Most flavoproteins do not have a NAD(P)H binding site. Instead, FAD is either oxidized or

reduced through its interaction with an electron transport flavoprotein. The acyl-CoA dehydrogenase protein family is not structurally related to FabK, but may provide clues to the FabK mechanism. Most of these enzymes catalyze the first step in fatty acid β-oxidation (the reverse reaction of FabI/K) [Thorpe and Kim, *FASEB J.*, **9**:718 (1995)]; however, a member of this family from *Ascaris* mitochondria actually carries out enoyl-CoA reduction (the FabI/K forward reaction) [Komuniecki *et al.*, *J. Biol. Chem.*, **260**:4770 (1985); Komuniecki *et al.*, *Biochim. Biophys. Acta*, **975**:127 (1989); and Duran *et al.*, *J. Biol. Chem.*, **268**:22391 (1993)]. These enzymes exist as homotetramers containing one molecule of FAD per subunit. A key feature of these soluble enzymes is that they interact with a low molecular weight flavoprotein, which in turn, shuttles reducing equivalents between the dehydrogenase/reductase and the membrane-associated electron transport chain [Thorpe and Kim, *FASEB J.*, **9**:718 (1995) and Komuniecki *et al.*, *J. Biol. Chem.*, **260**:4770 (1985); Komuniecki *et al.*, *Biochim. Biophys. Acta*, **975**:127 (1989); and Duran *et al.*, *J. Biol. Chem.*, **268**:22391 (1993)]. The unique FabK structure may permit the enoyl reductase step of fatty acid synthesis to receive reducing equivalents from either NADH or the electron transport chain depending on the energy balance of the cell.

A characteristic of the FabKs of the present invention is the following sequence motif:

P(I,V)XX(G,A)(G,P)MX{6,9}A(P,A,G,S,T)(P,A,G,S,T)V(S,A)XXGGX{22,28}T(Q,N,E,D)XPF(G,A)VX{90,105}P(V,I)(I,V)(A,G)(A,S)GGXXXXXXXXAX(F,L)XLGAXXXXXGTR (SEQ ID NO:45)

(1) Amino acids are defined using the one letter code, and "X" = any amino acid

(2) The first conserved prolyl residue is preferably 11 to 50 residues from the amino terminal residue

(3) Allowable substitutions at a particular position are in parenthesis: *i.e.* "(G, A)" means that a Gly or an Ala residue are found at that particular position.

(4) An "X { }" indicates the number (range) of the preceding residue is repeated and that any amino acid can be found in that span: *i.e.* X{6,9} indicates that there should be a span of 6 to 9 residues having any amino acid residue.

The motif can be defined as for "FindPatterns" in the GCG package . Using this pattern the only sequences found in the non-redundant database were those identified as FabK herein. All of the FabK proteins disclosed herein were identified as having this consensus sequence, whereas no other proteins were found to contain the consensus sequence. This consensus sequence can be depicted in the alternative manner: SEQ ID NO:45 A FabK Consensus Sequence

Pro Xaa_1 Xaa_2 Xaa_3 Xaa_4 Xaa_5 Met Xaa_6 Ala Xaa_7 Xaa_8 Val Xaa_9 Xaa_{10} Xaa_{11} Gly Gly Xaa_{12} Thr Xaa_{13}, Xaa_{14} Pro Phe Xaa_{15}Val Xaa_{16} Pro Xaa_{17} Xaa_{18} Xaa_{19} Xaa_{20} Gly Gly Xaa_{21} Xaa_{22} Xaa_{23} Xaa_{24} Xaa_{25} Xaa_{26} Xaa_{27} Xaa_{28} Ala Xaa_{29} Xaa_{30} Xaa_{31} Leu Gly Ala Xaa_{32} Xaa_{33} Xaa_{34} Xaa_{35} Xaa_{36} Gly Thr Arg

where: $\mathbf{Xaa_1}$ is Ile or Val, $\mathbf{Xaa_2}$ is any amino acid, $\mathbf{Xaa_3}$ is any amino acid, $\mathbf{Xaa_4}$ is Gly or Ala, $\mathbf{Xaa_5}$ is Gly or Pro, $\mathbf{Xaa_6}$ is at least 6 but less than 10 amino acids and they can be any amino acid, $\mathbf{Xaa_7}$ is Pro, Ala, Gly, Ser, or Thr, $\mathbf{Xaa_8}$ is Pro, Ala, Gly, Ser, or Thr, $\mathbf{Xaa_9}$ is Ser or Ala, $\mathbf{Xaa_{10}}$ is any amino acid, $\mathbf{Xaa_{11}}$ is any amino acid, $\mathbf{Xaa_{12}}$ is at least 22 but less than 29 amino acids and they can be any amino acid, $\mathbf{Xaa_{13}}$ is Gln, Asn, Glu, or Asp, $\mathbf{Xaa_{14}}$ is any amino acid, $\mathbf{Xaa_{15}}$ is Gly or Ala, $\mathbf{Xaa_{16}}$ is at least 90 but less than 106 amino acids and they can be any amino acid, $\mathbf{Xaa_{17}}$ is either Ile or Val, $\mathbf{Xaa_{18}}$ is either Ile or Val, $\mathbf{Xaa_{19}}$ is either Ala or Gly, $\mathbf{Xaa_{20}}$ is either Ala or Ser, $\mathbf{Xaa_{21}}$ is any amino acid, $\mathbf{Xaa_{22}}$ is any amino acid, $\mathbf{Xaa_{23}}$ is any amino acid, $\mathbf{Xaa_{24}}$ is any amino acid, $\mathbf{Xaa_{25}}$ is any amino acid, $\mathbf{Xaa_{26}}$ is any amino acid, $\mathbf{Xaa_{27}}$ is any amino acid, $\mathbf{Xaa_{28}}$ is any amino acid, $\mathbf{Xaa_{29}}$ is any amino acid, $\mathbf{Xaa_{30}}$ is either Phe or Leu, $\mathbf{Xaa_{31}}$ is any amino acid, $\mathbf{Xaa_{32}}$ is any amino acid, $\mathbf{Xaa_{33}}$ is any amino acid, $\mathbf{Xaa_{34}}$ is any amino acid, $\mathbf{Xaa_{35}}$ is any amino acid, and $\mathbf{Xaa_{36}}$ is any amino acid.

A consensus portion of FabK proteins without the flavin binding domain (FBD) that can be used to prepare antigenic fragments specific for FabK proteins is:

P(I,V)XX(G,A)(G,P)MX{6,9}A(P,A,G,S,T)(P,A,G,S,T)V(S,A)XXGG

X{22,28}T(Q,N,E,D)XPF(G,A)V (SEQ ID NO:46)

This consensus sequence can be depicted in the alternative manner: SEQ ID NO:46

Pro Xaa_1 Xaa_2 Xaa_3 Xaa_4 Xaa_5 Met Xaa_6 Ala Xaa_7 Xaa_8 Val Xaa_9 Xaa_{10} Xaa_{11} Gly Gly Xaa_{12} Thr Xaa_{13} Xaa_{14} Pro Phe Xaa_{15} Val

where **Xaa_1** is Ile or Val, **Xaa_2** is any amino acid, **Xaa_3** is any amino acid, **Xaa_4** is Gly or Ala, **Xaa_5** is Gly or Pro, **Xaa_6** is at least 6 but less than 10 amino acids and they can be any amino acid, **Xaa_7** is Pro, Ala, Gly, Ser, or Thr, **Xaa_8** is Pro, Ala, Gly, Ser, or Thr, **Xaa_9** is Ser or Ala, **Xaa_{10}** is any amino acid, **Xaa_{11}** is any amino acid, **Xaa_{12}** is at least 22 but less than 29 amino acids and they can be any amino acid, **Xaa_{13}** is Gln, Asn, Glu, or Asp, **Xaa_{14}** is any amino acid, and **Xaa_{15}** is Gly or Ala..

The present invention further includes the identification of another enoyl reductase, FabL: enoyl reductase III, that complements the growth of *E. coli* strain RJH13, (which contains a temperature-sensitive FabI), and that can confers complete triclosan resistance to *E. coli*. FabL has significant amino acid sequence similarity to FabI from *E. coli*, (about 30 % identical overall) indicating that FabI and FabL are both members of a larger family of proteins termed the short chain alcohol dehydrogenase/reductases (SDR). SDR proteins have approximately 30 % amino acid identity with each other. One key feature of an SDR that lacks enoyl reductase activity is the consensus amino acid sequence Tyr Xaa Xaa Xaa Lys (SEQ ID NO:61) in its active site. The corresponding SDRs that are enoyl reductases have the amino acid sequence Tyr Xaa Xaa Xaa Xaa Xaa Xaa Lys (SEQ ID NO:62) in their active site.

FabL has the enoyl reductase type consensus sequence suggesting that it may be an SDR enoyl reductase. However, such limited structural information alone cannot allow the identification since the overall identity of SDRs are low and the family size is large. Therefore, a nucleic acid encoding FabL was cloned from *B. subtilis* (prior gene designation from genomic sequencing project = ygaA; unidentified

dehydrogenase) and shown that it complements the growth of *E. coli*(fabI(Ts)) strain RJH13, and that it confers complete triclosan resistance to *E. coli*. These results demonstrate that FabL is indeed a FabI-like protein.

FabL proteins have the following consensus sequence:

G(A,G,S,P,T)(P,A,G,T,S)RG(I,V,L,M)GX{100,120}AQ(E,Q,N,D)AXKXMX {18,24}YXXXXXXKXA (V,I,L,M)E(T,A,S,P,G)XX(K,R,H)Y (SEQ ID NO: 57)

This consensus sequence can be depicted in the alternative manner:

SEQ ID NO: 57 A FabL Consensus Sequence

Gly Xaa_1 Xaa_2 Arg Gly Xaa_3 Gly Xaa_4 Ala Gln Xaa_5 Ala Xaa_6 Lys Xaa_7 Met Xaa_8 Tyr Xaa_9 Xaa_{10} Xaa_{11} Xaa_{12} Xaa_{13} Xaa_{14} Lys Xaa_{15} Ala Xaa_{16} Glu Xaa_{17} Xaa_{18} Xaa_{19} Xaa_{20} Tyr

where **Xaa_1** is either Ala, Gly, Ser, Pro, or Thr, **Xaa_2** is either Pro, Ala, Gly, Thr or Ser, **Xaa_3** is either Ile,Val, Leu, or Met, **Xaa_4** is at least 100 but less than 121 amino acids and they can be any amino acid, **Xaa_5** is either Glu,Gln, Asn or Asp, **Xaa_6** is any amino acid, **Xaa_7** is any amino acid, **Xaa_8** is at least 18 but less than 25 amino acids and they can be any amino acid, **Xaa_9** is any amino acid, **Xaa_{10}** is any amino acid **Xaa_{11}** is any amino acid **Xaa_{12}** is any amino acid **Xaa_{13}** is any amino acid **Xaa_{14}** is any amino acid, **Xaa_{15}** is any amino acid, **Xaa_{16}** is either Val, Ile, Leu, or Met, **Xaa_{17}** is either Thr ,Ala ,Ser, Pro, or Gly, **Xaa_{18}** is any amino acid, **Xaa_{19}** is any amino acid, and **Xaa_{20}** is either Lys, Arg, or His.

A region that can distinguish FabL from FabI is:

G(A,G,S,P,T)(P,A,G,T,S)RG(I,V,L,M)GX (SEQ ID NO:58)

This consensus sequence can be depicted in the alternative manner: SEQ ID NO:58

Gly Xaa_1 Xaa_2 Arg Gly Xaa_3 Gly Xaa_4

where **Xaa_1** is either Ala, Gly, Ser, Pro, or Thr, **Xaa_2** is either Pro, Ala, Gly, Thr, Ser **Xaa_3** is either Ile, Val, Leu, or Met, and **Xaa_4** is any amino acid.

The amino acid sequence SEQ ID NO:58 can be used to make antigenic fragments that are specific for FabL proteins for example.

Unlike, FabK proteins, certain FabL proteins have been previously described but their activity as enoyl reductases have not been disclosed for the most part. Thus the *Helicobacter* FabL protein has been described in the context of its similarity to known genes only, as an α-hydroxysteroid dehydrogenase (a member of the SDR family), and has not been characterized biochemically. The lone exception may be the Streptomyces FabL protein which was identified as a NADPH-dependent 1-cyclohexenylcarbonyl CoA reductase [Wang *et al.*, *J. Bacteriol.* **178 (23)**, 6873-6881 (1996)]. The protein is has been reported to be involved in the conversion of shikimic acid to cyclohexanecarboxylic acid, which is used for cyclohexyl fatty acid biosynthesis and polyketide (ansatrienin) biosynthesis.

Table 3

Identification of Enoyl Reductases from Assorted Unicellular Organisms

Organism	Nucleic Acid	Amino Acid
S. pneumoniae	SEQ ID NO:1	SEQ ID NO:2
S. mutans	SEQ ID NO:3	SEQ ID NO:4
S. pyogenes	SEQ ID NO:5	SEQ ID NO:6
E. faecalis	SEQ ID NO:9	SEQ ID NO:10
C. acetobutylicum	SEQ ID NO:11	SEQ ID NO:12
C. difficile	SEQ ID NO:13	SEQ ID NO:14
P. gingivalis	SEQ ID NO:15	SEQ ID NO:16
Ca. Cresentus	SEQ ID NO:17	SEQ ID NO:18
Ps. Aeruginosa	SEQ ID NO:19	SEQ ID NO:20
Mycobacterium tuberculosis rv3553	SEQ ID NO:27	SEQ ID NO:28
Mycobacterium tuberculosis rv0021c	SEQ ID NO:29	SEQ ID NO:30
T. maritima	SEQ ID NO:33	SEQ ID NO:34
H. pylori	SEQ ID NO:35	SEQ ID NO:36
A. fulgidis	SEQ ID NO:37	SEQ ID NO:38
Consensus Sequence (*plus* *FBD)		SEQ ID NO:45
Consensus Sequence (*minus* FBD)		SEQ ID NO:46
St. aureus NCTC	SEQ ID NO:47	SEQ ID NO:48
Bacillus subtilis (FabL)	SEQ ID NO:49	SEQ ID NO:50
Campylobacter jejuni (FabL)	SEQ ID NO:51	SEQ ID NO:52
Helicobacter pylori (FabL)	SEQ ID NO:53	SEQ ID NO:54
Streptomyces collinus FabL	SEQ ID NO:55	SEQ ID NO:56
Consensus Sequence (FabL)		SEQ ID NO:57
Consensus Sequence (FabL)		SEQ ID NO:58

*FBD is short for the flavin binding domain.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.